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(54) Title: METHODS AND COMPOSITIONS FOR ISOLATION OF NUCLEIC ACID MOLECULES

(57) Abstract: The present invention relates generally to compositions, methods and kits for use in isolating nucleic acid molecules. More specifically, the invention relates to such compositions, methods and kits that are useful in the isolation of nucleic acid molecules from cells (e.g., bacterial cells, animal cells, fungal cells, viruses, yeast cells or plant cells) via lysis and one or more additional isolation steps, such as one or more chromatography steps. In particular, the invention relates to compositions, methods and kits wherein nucleic acid molecules are isolated using an integrated lysis/chromatography matrix, which may comprise one or more supports (e.g., polyolefin, scintered polyethylene, nitrocellulose, polypropylene, polycarbonate, cellulose acetate, silica, and the like) that has been treated with or associated with one or more chemical lysis reagents (e.g., one or more detergents, one or more chaeotropes, one or more enzymes, and the like). The compositions, methods and kits of the invention are suitable for isolating a variety of forms of nucleic acid molecules from cells, including but not limited to plasmids, vectors, DNA, cDNA, RNA, mitochondrial DNA, chloroplast DNA, and the like, any of which may be single-stranded, double-stranded, linear or circular. The compositions, methods and kits of the invention are particularly well-suited for rapid isolation of soluble plasmid or vector DNA from bacterial cells.

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Methods and Compositions for Isolation of Nucleic Acid Molecules

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is in the fields of molecular biology and genetics. The invention relates generally to compositions, methods and kits for use in isolating nucleic acid molecules. More specifically, the invention relates to such compositions, methods and kits that are useful in the isolation of small molecular weight nucleic acid molecules (e.g., vectors, plasmids, and the like) from cells via lysis and one or more additional isolation steps, such as one or more chromatography steps. The compositions, methods and kits of the invention are suitable for isolating a variety of forms of nucleic acid molecules from cells.

Related Art

The most popular method of plasmid DNA isolation from bacterial cells is based on alkaline lysis, followed by batch chromatography. In this lysis procedure (Birnboim, H. and Doly, J. (1979) *Nucleic Acids Res.* 7, 1513) cells are completely ruptured, released proteins and nucleic acids are denatured, then plasmid DNA is preferentially renatured. Precipitated denatured proteins and chromosomal DNA are then separated from soluble plasmid DNA. Typically, plasmid DNA is further purified from residual contaminating proteins, lipids and nucleic acids by selective binding and release from a chromatography matrix. The chromatography matrix, in one instance, is an anion-exchange resin, e.g., Qiagen-tip 20 (Qiagen, and U.S. Patent No. 4,997,932) or CONCERT High Purity Plasmid Miniprep System (Life Technologies, Inc. and U.S. Patent No. 5,843,312). Alternatively, the chromatography matrix is a silica adsorption resin, such as Wizard Minipreps DNA Purification Resin (Promega, and U.S. Patent No. 5,658,548) or CONCERT Rapid Plasmid Miniprep System (Life Technologies,

Inc.). Although effective for the purification of plasmid DNA, several deficiencies in the alkaline lysis method are recognized: first, it represents a relatively long processing time, second, it includes many manual manipulations with at least three different solutions, third, there is a necessary removal of the precipitate prior to chromatography, and fourth, there is a requirement at several steps for careful manipulation by an experienced operator.

Recently, a new type of system was introduced for the purification of genomic, DNA from eukaryotic cells and of chromosomal DNA from Gram-negative bacteria. In the GENERATION DNA Purification Capture Column Kit (Gentra Systems), a purification matrix is used to lyse cells and capture the DNA. Trapped DNA is washed first with buffer, then is released by heating the matrix in Elution Solution for 10 minutes at 99°C, followed by centrifugation. This system combines cell lysis and solid phase chromatography into a single operation, similar to the method of Boom *et al* (U.S. Patent No. 5,234,809). This method is distinct from Boom *et al* in that DNA probably is trapped by the matrix fibers and only released upon denaturation, causing the genomic DNA to be converted into fragmented, single strands. Upon cell lysis in the Boom *et al* method, DNA is adsorbed immediately onto silica particles, which are then washed by a separate filtration operation. DNA is removed from the silica by filtration following application of a low salt buffer at room temperature. The gentle elution conditions maintains native DNA structure, giving the DNA broader utility and is preferred over denatured DNA derived from the GENERATION Kit.

Despite the availability in the art of techniques for isolation of genomic and chromosomal DNA, however, there remains a need in the art for rapid and efficient compositions, methods and kits useful in the isolation of small molecular weight nucleic acid molecules, such as plasmids, vectors, and the like. The present invention provides such compositions, methods and kits.

BRIEF SUMMARY OF THE INVENTION

The present invention relates generally to compositions, methods and kits for use in isolating nucleic acid molecules. More specifically, the invention relates to such compositions, methods and kits that are useful in the isolation of nucleic acid molecules from cells (e.g., bacterial cells, animal cells, fungal cells, yeast cells or plant cells) via lysis and one or more additional isolation steps, such as one or more chromatography steps. In particular, the invention relates to compositions, methods and kits wherein desired nucleic acid molecules are isolated using an integrated lysis/chromatography matrix.

More particularly, the invention relates to methods for isolating nucleic acid molecules, particularly low molecular weight nucleic acid molecules, comprising:

(a) providing one or more cells or a cellular source containing low molecular weight nucleic acid molecules;

(b) contacting the one or more cells or cellular source with at least one pore-containing matrix which binds or traps high molecular weight nucleic acid molecules (e.g., genomic or chromosomal nucleic acid molecules) but does not substantially bind or trap low molecular weight nucleic acid molecules (e.g., vectors, plasmids, etc.);

(c) causing the one or more cells or cellular source (or portion thereof) to lyse or disrupt (e.g., disrupt the integrity of the cell membrane and/or cell wall) such that low and high molecular weight nucleic acid molecules are released from the one or more cells or cellular source; and

(d) collecting the low molecular weight nucleic acid molecules.

In accordance with the invention, the cells may be lysed or disrupted before contacting the cells with the matrix, although cell lysis or disruption preferably takes place after the cells are contacted with the matrix and more preferably at the same time or approximately the same time (e.g., simultaneously

or substantially simultaneously) the cells are contacted with the matrix. In another aspect, the cells are preferably trapped within or on the matrix prior to or during cell lysis or disruption. In yet another aspect, the cells are lysed/disrupted by contacting them with a composition or compound which causes or aids in cell lysis or disruption, although mechanical or physical forces (e.g., pressure, sonication, temperature (heating, freezing), and/or freeze-thawing etc.) may be used in accordance with the invention. In addition, any combination of mechanical forces, physical forces or lysis compositions/compounds may be used to disrupt/lyse the cells. After the one or more cells (or portion thereof) are lysed/disrupted in accordance with the invention, the low molecular weight nucleic acid molecules (or portion thereof) are substantially separated from the high molecular weight nucleic acid molecules. Such separation is preferably accomplished by the matrix binding/trapping the high molecular weight molecules and not substantially trapping/binding the low molecular weight molecules. Such action allows physical separation of such molecules where the smaller molecules of interest are allowed to substantially pass through the matrix while the larger molecules are trapped or bind to the matrix.

According to the invention, the matrix may be any porous matrix that traps or binds high molecular weight and/or does not substantially bind or trap low molecular weight nucleic acid molecules. Such matrix may include but is not limited to a polyester matrix, a polyolefin matrix, a scintered polyethylene matrix, a nitrocellulose matrix, a cellulose acetate matrix, a cellulose matrix, a porous ceramic matrix, a silica matrix, a polysaccharide matrix (Sepharose, agarose, Sephadex, etc.), a polymer matrix (Sephacryl, Trisacryl, Toyopearl, Bio-Gel, etc.) and the like. In a preferred aspect, the matrix is a solid matrix, although the matrix may be a semi-solid matrix. Suitable matrix materials may be obtained commercially, for example from Filtrona Richmond, Inc. (Richmond, Virginia), Bio-Rad (Richmond, California), Gentra Systems (Minneapolis, MN), Tosohaas (Montgomeryville, PA), BioSepra, Inc., (Marlborough, MA), and Porex Technologies Corp. (Fairburn, GA). In a related aspect, the matrix may be prepared in various sizes, shapes, and forms including flat, wafer, cylindrical,

rectangular, beads, gels, square, cartridge, swab tip, plug, frit, membrane and the like, and may also be contained in various containers such as tubes, bottles, vials, ampules, microspin tubes, wells, multi-well plates, bags and the like. In a preferred aspect, the invention involves the use of size separation chromatography and/or filtration to separate or substantially separate low molecular weight nucleic acid molecules (e.g., vectors) from high molecular weight nucleic acid molecules. Thus, any matrix which provides desired size separation (e.g., filters, chromatography supports, etc.) may be used in the invention. One of skill in the art can readily determine the appropriate matrix, pore size of the matrix, size, shape and dimensions of the matrix taking into consideration the type and size of the desired nucleic acid molecules, the cell type or cellular source, the size of the undesired nucleic acid molecules etc. In another aspect, the invention combines such size separation chromatography/filtration with cell lysis/disruption (preferably such lysis/disruption is done when or approximately when the cellular source comes in contact with or after the cellular source is in contact with the chromatography/filtration matrix). The pores or passage ways in the matrix are typically small enough to prevent passage of large nucleic acid molecules but large enough to permit passage of low molecular weight nucleic acid molecules, and may range from about 0.1 to about 10,000 micrometers in diameter, about 0.1 to about 5,000 micrometers in diameter, about 0.1 to about 1,000 micrometers in diameter, about 1 to about 500 micrometer in diameter, about 10 to about 500 micrometer in diameter, or about 25 to about 400 micrometers in diameter.

In one preferred embodiment, the composition or compound that disrupts the cellular membrane or cell wall integrity may comprise one or more detergents, such as sodium dodecylsulfate (SDS) or Sarkosyl, Triton X-100, NP-40, deoxycholate or Brij 35; one or more chaeotropic agents such as sodium iodide, sodium perchlorate or guanidine or a salt thereof; one or more enzymes such as zymolyase, lyticase, lysozyme or lysostaphin; one or more inorganic salts such as sodium chloride, potassium chloride, or lithium chloride; one or more acids and/or bases or buffering agents (e.g., to increase or reduce pH); one or more organic solvents such as toluene, phenol, ethanol, isopropanol, isoamyl alcohol, butanol,

an ether (e.g., diethyl ether, dimethyl ether, or ethylmethyl ether), or chloroform; or any other compound or enzyme which disrupts the integrity of (i.e., lyses or causes the formation of pores in) the cell membrane and/or cell walls (e.g., polymixin B). In another aspect, the composition may comprise one or more compounds or enzymes to degrade, destroy or remove unwanted components or contaminants (e.g., ribonucleases (RNases) to remove or destroy or degrade undesired RNA released from the cellular source and/or proteases (e.g., Proteinase K) to destroy or degrade released proteins). The compositions may also include any combination of the above described components. In one particularly preferred such aspect, the composition may be adsorbed onto or complexed with or associated with the matrix prior to applying the one or more cells or cellular source to the matrix. In a preferred aspect, the composition is dried in or on the matrix. Thus, in a preferred aspect, the matrix comprises a cell lysis/disruption compound or composition. In this aspect, the cell disruption/lysis may occur when or about the same time the cells come into contact with the composition containing matrix. In another aspect, the composition is added after the cells are added to (e.g., bound to or associated with) the matrix. In yet another aspect, the composition is added to the cells prior to adding the cells to the matrix. In this aspect, the composition may be formulated to weaken the cell membrane/cell wall such that the cells will substantially disrupt/lyse when contacted with the matrix. Alternatively, the composition will substantially lyse/disrupt the cells before addition to the matrix.

In accordance with the invention, the nucleic acid molecules of interest (e.g., plasmids, vectors, etc.) may be removed from the matrix by elution with an aqueous solution, such as a buffered salt solution or elution buffer. The unwanted molecules (e.g., chromosomal or genomic DNA) are substantially retained in or on the matrix, thus allowing the desired nucleic acid molecules to be eluted or to be substantially removed from the matrix. Such elution or removal of the desired nucleic acid molecules may be facilitated by centrifugation, gravity, vacuum, pressure, etc., which provides flow of the desired nucleic acid sample from the matrix. The isolated nucleic acid molecules of interest may then be further

purified by standard nucleic acid purification techniques and/or further manipulated by standard molecular biology techniques such as sequencing, amplification, endonuclease digestion (e.g., restriction enzyme digestion), nucleic acid synthesis, transformation, transfection, and the like.

The methods according to the invention are suitable for isolation of low molecular weight nucleic acid molecules from any cell or cellular source, including bacterial cells (particularly *Escherichia coli* cells), yeast cells, fungal cells, animal cells (particularly insect cells, and mammalian cells including human cells, CHO cells, VERO cells, Bowes melanoma cells, HepG2 cells, and the like), and plant cells, any of which may be transformed cells, established cell lines, cancer cells, or normal cells. The methods of the invention are particularly well-suited for isolation of extrachromosomal nucleic acid molecules, including but not limited to plasmids, vectors, phagemids, cosmids, BACs, PACs, YACs, cDNA molecules or cDNA libraries, mitochondrial nucleic acid molecules, and chloroplast nucleic acid molecules, any of which may be single-stranded or double-stranded, linear or circular, supercoiled, and which may be DNA or RNA molecules.

The invention also relates to isolated nucleic acid molecules produced by the methods of the invention, which are preferably low molecular weight nucleic acid molecules, such as the extrachromosomal nucleic acid molecules described herein and particularly plasmids and vectors. The invention also relates to vectors (particularly expression vectors) and host cells comprising these isolated nucleic acid molecules of the invention. The invention also relates to further manipulation of the isolated nucleic acid molecules of the invention by standard molecular biology techniques such as sequencing, nucleic acid synthesis, cloning, amplification, endonuclease digestion (restriction endonuclease digestion), transformation, transfection and the like.

In a related aspect, the invention relates to compositions for use in isolating low molecular weight nucleic acid molecules. Such compositions of the invention preferably comprise one or more components, such as:

(a) a cellular source of the low molecular weight nucleic acid molecules of interest;

(b) a nucleic acid-binding portion comprising at least one pore-containing matrix which binds or traps high molecular weight nucleic acid molecules but not substantially trap or bind low molecular weight nucleic acid molecules; and

(c) a cell disrupting or cell lysis portion comprising at least one compound that disrupts the integrity of the cellular membrane or cell wall when the cellular source comes into contact with said compound.

Preferred cellular sources, solid matrices, and lysis/disrupting compounds for use in the compositions of the invention include those described and used in the methods of the present invention. In a preferred composition of the invention, the compound that disrupts the integrity of the cellular membrane and/or cell wall is adsorbed onto or complexed with or associated with the matrix, for example by ionic, hydrophobic, or covalent or non-covalent attachment of the cell membrane/cell wall disrupting compound to the matrix material. In a preferred aspect, such compound is dried in or on the matrix. The compositions of the invention are useful in isolating a variety of low molecular weight nucleic acid molecules, particularly those described herein and most particularly plasmids and vectors.

The invention also relates to kits for use in isolating low molecular weight nucleic acid molecules, comprising one or more of the components for carrying out the methods of the invention or one or more of the compositions of the invention. Such kits of the invention may comprise one or more components, which may be contained in one or more containers such as boxes, cartons, tubes, vials, ampules, bags, and the like. In one such aspect, the kits of the invention may comprise:

(a) at least one pore-containing matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules (and which preferably traps a cellular source of high and

low molecular weight nucleic acid molecules within or on the matrix); and

(b) a cell disrupting/lysing composition comprising at least one compound that disrupts the integrity of the cellular membrane or cell wall when the cellular source comes into contact with the compound or composition, such that the high and low molecular weight nucleic acid molecules (or portion thereof) are released from the cellular source.

In one such kit, the matrix comprises the cell disrupting/lysing composition or compound. Such cell disrupting/lysing composition or compound may be adsorbed onto or complexed with or associated with the matrix, for example by ionic, hydrophobic, non-covalent or covalent attachment to the matrix material. Such cell disrupting/lysing composition is preferably dried in or on the matrix. Preferred solid matrix materials, cell disrupting/lysing compositions and compounds, and washing and elution compositions for use in the kits of the invention include those described herein for use in the methods of the present invention. In related aspects, the kits of the invention further comprise one or more additional reagents, such as one or more restriction enzymes, one or more polypeptides having nucleic acid polymerase activity (e.g., one or more DNA polymerases which may be thermostable DNA polymerases and/or one or more reverse transcriptases which may be substantially reduced in RNase H activity), one or more cells competent for transformation and/or other transformation (e.g., competent cells) transfection reagents (e.g., cationic lipids) or other components or reagents that may be useful in conjunction with further purification, processing and analysis of the isolated nucleic acid molecules of the invention, for example, components or reagents useful in nucleic acid amplification, sequencing, cloning, transfection, transcription, translation, and the like. Such kits of the invention may also comprise protocols or instructions for carrying out the methods of the invention.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of what is known in the art, the following drawings and description of the invention, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of one aspect of the invention, depicting a thin-walled tube (preferably a microfuge tube of any size) **1** containing a porous, matrix material in the form of a frit or plug or cartridge or swab tip **2** which divides the airspace within the tube into an upper sample application section **3** and a lower sample collection or sample elution section **4**. According to one aspect of the invention, the matrix material **2** may comprise one or more cell disrupting/lysing compounds or compositions. In another aspect, the matrix material may be in the form of beads or a gel or other semi-solid matrix in which case the matrix is preferably encased or associated with a solid support material to maintain the upper sample-application section **3** and the lower sample collection section **4**. Preferably, the matrix material (solid or semi-solid) is in the form of a cartridge or plug or swab tip which can be easily removed from the tube **1** to facilitate sample collection. In another aspect, one or more additional matrices or resins may be included in the upper sample application section **3** and/or in the sample-collection section **4**, to further facilitate isolation or purification of the desired nucleic acid molecules. For example, well known nucleic acid binding matrices (such as silica adsorption resins, anion-exchange resins, reverse phase resins, and/or affinity resins) may be included below a size separation matrix of the invention to further remove or substantially remove undesired components including proteins, lipids, lysis/disruption compositions used to lyse/disrupt the cellular source, solvents, detergents, etc. Alternatively, matrices (such as cation-exchange resins, reverse phase resins, and/or hydrophobic interaction resins) which bind undesired such components but which do not substantially bind the desired nucleic acids may be used. In another embodiment, combinations of such nucleic acid binding matrices and contaminant binding matrices may be used. The optional nucleic acid binding

resin and/or contaminant binding resin 5 is shown. Such additional matrices are preferably in cartridge or plug or swab tip form. In another aspect, the sample-collection section 4 may contain an opening or access port (which may be closed if desired) to collect samples without the need to remove the matrix or matrices. In one example, where a size separation matrix and a nucleic acid binding matrix are provided, the desired nucleic acid molecules pass through the size separation matrix and bind to the binding matrix. Then be applied to remove unwanted materials through the access port or opening within the sample-collection section 4. If desired, prior to the addition of the wash buffers, the seze separation matrix may be removed from the tube 1. The desired isolated nucleic acid molecules may then be removed from the access port/opening when an elution buffer is applied. Alternatively, the removal of desired nucleic acid molecules is accomplished by removal of the matrix or matrices to access the sample-collection section 4.

Figure 2 is a photograph of an ethidium bromide-stained 1% agarose gel, analyzing samples of nucleic acid molecules at various stages of isolation by the methods of the invention. Lane 1: 10µl of flow through from swab tip (Sample 1); lane 2: 10µl of trapped DNA released from swab tip (Sample 2); lane 3: 10µl of eluant from Concert Rapid spin cartridge (Sample 3); lane 4: Purified plasmid pRPA-1; lane 5: 10µl of Sample 1 digested with 10 units of *Hin*d III; lane 6: 10µl of Sample 3 digested with 10 units of *Hin*d III; lane 7: Purified pRPA-1 digested with 10 units of *Hin*d III; M: Molecular weight markers — Supercoiled DNA Ladder (left) and 1 Kb DNA Ladder (right).

Figure 3 is a diagram of one aspect of the invention, depicting a thin-walled tube or column (preferably microspin or spin cartridges of any size) 1 containing a size separation matrix (for separating large nucleic acid molecules from small nuclei acid molecules) 2 and a second matrix material 5 for further purifying small molecular weight nucleic acid molecules. Preferably, the matrix material 5 is a nucleic acid binding matrix or a contaminant binding matrix, or combinations thereof. The size separation matrix 2 and the preferred nucleic acid binding matrix 5 may be separated by a space within the tube or column 1,

although such matrices are preferably in close proximity and preferably separated by a frit or other material. The tube of column 1 contains a sample application section 3 and an opening or access port 6 (which may be closed if desired) to collect the sample. An optional collection tube, well or container 7 is provided for collecting samples passing through the opening or access port 6. In a preferred aspect, the size separation matrix 2 comprises a cell lysis/disruption compound or composition. In the application of such preferred embodiment, a sample containing a cellular source of large and small molecular weight nucleic acid molecules are applied to the sample application section 3 preferably to the upper surface of the matrix 2. The cell lysis/disruption composition or compound causes release of the low and/or high molecular weight nucleic acid molecules which separate according to size in the size separation matrix 2, allowing low molecular weight nucleic acid molecules to pass through the matrix 2, while a substantial portion of the large molecular weight nucleic acid molecules are retained in or on the matrix 2. Small molecular weight nucleic acid molecules passing through the size separation matrix 2 bind to the nucleic acid binding matrix 5. The size separation matrix 2 may optionally be removed from the column or tube 1 (before or after washing) to minimize large molecular weight nucleic acid molecules from passing through the size separation matrix 2 during subsequent washing and elution steps. Washing buffers or solutions may then be applied to remove unwanted materials. An elution buffer or solution may then be applied to elute the desired low molecular weight nucleic acid molecules from the nucleic acid binding matrix and through the opening or access port 6. During washing, the collection tube 7 (containing the unwanted materials) can be replaced with a second or new collection tube 7 to collect the desired nucleic acid molecules upon elution.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions, methods, and kits that may be used in isolating nucleic acid molecules from a nucleic acid-containing sample. It will be readily appreciated by those skilled in the art that, in accordance with the

present invention, any cell, tissues, organs, populations of cells, etc. can be used as a nucleic acid source. Preferably, such nucleic acid sources are used to isolate any given plasmid, vector, or other extragenomic or extrachromosomal structure. In a particular aspect, a population of different vectors (for example, a cDNA library or genomic library contained by the cell population or culture) is used as a nucleic acid source. Thus, the invnetion allows isolation of such population of vectors/cDNA library/genomic library.

In the description that follows, a number of terms used in the fields of molecular biology and recombinant DNA technology are utilized extensively. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Low molecular weight nucleic acid molecule. As used herein, the phrase "low molecular weight nucleic acid molecule" refers to any nucleic acid molecule or population of nucleic acid molecules which is smaller in size or molecular mass than a genomic nucleic acid molecule such as a chromosome from a given cell or cellular source. Low molecular weight nucleic acid molecules according to the invention typically are no larger than about 500 kilobases in size, and are preferably about 1 to about 300, more preferably about 1 to about 200, more preferably about 1 to about 100, still more preferably about 1 to about 50, still more preferably about 1 to about 25 and most preferably about 1 to about 15 kilobases in size. In contrast, the term "high molecular weight nucleic acid molecule" is used to mean any nucleic acid molecule or population of nucleic acid molecules that is larger than about 500 kilobases in size, particularly genomic nucleic acid molecules or chromosomes which may range in size from about 1 to about 15,000 megabases. Examples of low molecular weight nucleic acid molecules include, but are not limited to, plasmids, large molecular weight plasmids (BAC's, PAC's, YAC's), vectors, phagemids, cosmids, mitochondrial nucleic acid molecules, chloroplast nucleic acid molecules, cDNA molecules or cDNA libraries, amplification fragments (e.g., PCR generated nucleic acid molecules) and fragments of nucleic acid molecules regardless of the means by

which such fragments are generated. Such fragments may be generated by enzymatic digestion (e.g., endonuclease digestion for example with type I and/or II restriction enzymes), mechanical forces (shearing) and the like. In particular, fragments of genomic or chromosomal nucleic acid molecules which are smaller in size than the complete genomic or chromosomal nucleic acid molecule from which they are derived are included in this definition.

Amplification. As used herein, "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a nucleic acid (e.g., DNA) molecule or primer thereby forming a new nucleic acid molecule complementary to the nucleic acid template. The formed nucleic acid molecule and its template can be used as templates to synthesize additional nucleic acid molecules. As used herein, one amplification reaction may consist of many rounds of nucleic acid synthesis. Amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a nucleic acid molecule.

Host. Any prokaryotic or eukaryotic cell that is the recipient of a replicable expression vector or cloning vector. The terms "host" or "host cell" may be used interchangeably herein. For examples of such hosts, see Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Preferred prokaryotic hosts include, but are not limited to, bacteria of the genus *Escherichia* (e.g., *E. coli*), *Bacillus*, *Staphylococcus*, *Agrobacter* (e.g., *A. tumefaciens*), *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, *Caryophanon*, etc. The most preferred prokaryotic host is *E. coli*. Bacterial hosts of particular interest in the present invention include *E. coli* strains K12, DH10B, DH5α and HB101. Preferred eukaryotic hosts include, but are not limited to, fungi, fish cells, yeast cells, plant cells and animal cells. Particularly preferred animal cells are insect cells such as *Drosophila* cells, *Spodoptera* Sf9, Sf21 cells and *Trichoplusa* High-Five cells; nematode cells such

as *C. elegans* cells; and mammalian cells such as COS cells, CHO cells, VERO cells, 293 cells, PERC6 cells, BHK cells and human cells. In accordance with the invention, a host or host cell may serve as the cellular source for the desired nucleic acid molecule to be isolated.

Vector. A vector is a nucleic acid molecule (preferably DNA) capable of replicating autonomously in a host cell. Such vectors may also be characterized by having a small number of endonuclease restriction sites at which such sequences may be cut without loss of an essential biological function and into which nucleic acid molecules may be spliced to bring about its replication and cloning. Examples include plasmids, autonomously replicating sequences (ARS), centromeres, cosmids and phagemids. Vectors can further provide primer sites, e.g., for PCR, transcriptional and/or translational initiation and/or regulation sites, recombinational signals, recombination sites (see, for example, co-pending U.S. application serial no. 08/663,002 filed June 7, 1996 and U.S. application serial no. 60/065,930 filed October 24, 1997), replicons, etc. The vector can further contain one or more selectable markers suitable for use in the identification of cells transformed or transfected with the vector, such as kanamycin, tetracycline, amplicillin, etc.

In accordance with the invention, any vector may be used. In particular, vectors known in the art and those commercially available (and variants or derivatives thereof) may be used in accordance with the invention. Such vectors may be obtained from, for example, Vector Laboratories Inc., InVitrogen, Promega, Novagen, NEB, Clontech, Boehringer Mannheim, Pharmacia, EpiCenter, OriGenes Technologies Inc., Stratagene, Perkin Elmer, Pharmingen, Life Technologies, Inc., and Research Genetics. Such vectors may be used for cloning or subcloning nucleic acid molecules of interest and therefore recombinant vectors containing inserts, nucleic acid fragments or genes may also be isolated in accordance with the invention. General classes of vectors of particular interest include prokaryotic and/or eukaryotic cloning vectors, expression vectors, fusion vectors, two-hybrid or reverse two-hybrid vectors, shuttle vectors for use in different hosts, mutagenesis vectors, transcription vectors, vectors for receiving

large inserts (yeast artifical chromosomes (YAC's), bacterial artificial chromosomes (BAC's) and P1 artifical chromosomes (PAC's)) and the like. Other vectors of interest include viral origin vectors (M13 vectors, bacterial phage λ vectors, baculovirus vectors, adenovirus vectors, and retrovirus vectors), high, low and adjustable copy number vectors, vectors which have compatible replicons for use in combination in a single host (e.g., pACYC184 and pBR322) and eukaryotic episomal replication vectors (e.g., pCDM8). The vectors contemplated by the invention include vectors containing inserted or additional nucleic acid fragments or sequences (e.g., recombinant vectors) as well as derivatives or variants of any of the vectors described herein.

Expression vectors useful in accordance with the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker (such as a tetracycline or ampicillin resistance genes) and one or more promoters such as the phage lambda P_L promoter, and/or the *E. coli lac*, *trp* and *tac* promoters. Other suitable promoters will be known to the skilled artisan.

Among vectors preferred for use in the present invention include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from Invitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2 and pSV·SPORT1, available from Life Technologies, Inc. Other suitable vectors will be readily apparent to the skilled artisan.

Plasmid. As used herein, the term plasmid means an extrachromosomal genetic element, typically less than about 25 kilobases (kb) in size and more typically about 15 kb to about 2 kb in size.

Isolated. As used herein, the term "isolated" (as in "isolated nucleic acid molecule") means that the isolated material, component, or composition has been

at least partially purified away from other materials, contaminants, and the like which are not part of the material, component, or composition that has been isolated. For example, an "isolated low molecular weight nucleic acid molecule" is a nucleic acid molecule that has been treated in such a way as to remove at least some of the other nucleic acid molecules (e.g., large nucleic acid molecules) with which it may be associated in the cell, tissue, organ or organism. In particular, the term "isolated low molecular weight nucleic acid molecule" or "isolated vector" refers to a low molecular weight nucleic acid molecule preparation or vector preparation which contains no more than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, and 7%, preferably no more than 5%, 2.5%, and 2%, and most preferably less than 1%, 0.5%, and 0.1% (percentages by weight) of high molecular weight nucleic acid molecules (e.g., chromosomal/genomic DNA). As one of ordinary skill will appreciate, however, a solution comprising an isolated nucleic acid molecule may comprise one or more buffer salts and/or a solvents, e.g., water or an organic solvent such as acetone, ethanol, methanol, and the like, and yet the nucleic acid molecule may still be considered an "isolated" nucleic acid molecule with respect to its starting materials.

Cell disrupting or cell lysing compound or composition. As used herein, "cell disrupting" or "cell lysing" refers to a composition or a component of a composition that effects lysis, rupture, or poration of the cells, tissues, or organisms used as the source of the nucleic acid molecules to be isolated, such that the nucleic acid molecules (or portion thereof) that are contained in the cell, tissue, or organism source are released from the cell, tissue, or organism. According to the invention, the cells, tissues, or organisms need not be completely lysed, ruptured or porated, and all of the nucleic acid molecules contained in the source cells, tissues or organisms need not be released therefrom. Preferably, a cell disrupting or cell lysis compound or composition at least 25%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or more of the total nucleic acid molecules, particularly the total low molecular weight nucleic acid molecules (such as vectors, plasmids, and the like) that are contained in the cell, tissue, or organism.

Other terms used in the fields of recombinant DNA technology and molecular and cell biology as used herein will be generally understood by one of ordinary skill in the applicable arts.

Sources of DNA

The methods, compositions and kits of the invention are suitable for isolation of low molecular weight nucleic acid molecules from any cellular source, including a variety of cells, tissues, organs or organisms, which may be natural or which may be obtained through any number of commercial sources (including American Type Culture Collection (ATCC), Rockville, Maryland; Jackson Laboratories, Bar Harbor, Maine; Cell Systems, Inc., Kirkland, Washington; Advanced Tissue Sciences, La Jolla, California). Cells that may be used as cellular nucleic acid sources may be prokaryotic (bacterial, including members of the genera *Escherichia* (particularly *E. coli*), *Serratia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Chlamydia*, *Neisseria*, *Treponema*, *Mycoplasma*, *Borrelia*, *Bordetella*, *Legionella*, *Pseudomonas*, *Mycobacterium*, *Helicobacter*, *Agrobacterium*, *Collectotrichum*, *Rhizobium*, and *Streptomyces*) or eukaryotic (including fungi or yeasts, plants, protozoans and other parasites, and animals including humans and other mammals). Any virus may also be used as a cellular source of nucleic acid molecules in accordance with the invention. Also suitable for use as sources of low molecular weight nucleic acid molecules are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus. These cells, tissues and organs may be normal, transformed, or established cell lines, or they may be pathological such as those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS) or parasites), in genetic or biochemical pathologies (e.g., cystic fibrosis, hemophilia, Alzheimer's disease, schizophrenia, muscular dystrophy or multiple sclerosis), or in cancers and cancerous processes. The methods, compositions and kits of the invention are particularly well-suited for isolation of

extrachromosomal nucleic acid molecules, including but not limited to plasmids, vectors, phagemids, cosmids, cDNA molecules, mitochondrial nucleic acid molecules, and chloroplast nucleic acid molecules, any of which may be single-stranded or double-stranded, linear or circular, supercoiled, and which may be DNA or RNA molecules. In a particularly preferred aspect, the methods of the invention are useful in the isolation of plasmid or vector DNA from bacterial cells. Other cells, tissues, viruses, organs and organisms that will be familiar to one of ordinary skill in the art may also be used as sources of nucleic acid molecules for the preparation of isolated nucleic acid molecules according to the present invention.

Methods

In one aspect, the invention relates to methods for isolating nucleic acid molecules, particularly small molecular weight nucleic acid molecules such as plasmids, vectors, organellar nucleic acid molecules, and the like. Methods according to this aspect of the invention may comprise one or more steps which result in the isolation of one or more nucleic acid molecules or populations of nucleic acid molecules (e.g., a cDNA library) from the natural environment in which the nucleic acid molecules are found.

In one preferred such aspect, the methods of the invention may comprise:

(a) contacting a cellular source of nucleic acid molecules, with at least one pore-containing matrix which binds or traps high molecular weight nucleic acid molecules (e.g., genomic or chromosomal nucleic acid molecules) but does not substantially bind or trap low molecular weight nucleic acid molecules and causing the cellular source (or portion thereof) to release all or a portion of the desired low molecular weight nucleic acid molecules; and

(b) separating or substantially separating the low molecular weight nucleic acid molecules from the high molecular weight nucleic acid molecules.

More specifically, the invention relates to a method for obtaining one or more vectors comprising:

(a) contacting a cellular source of one or more vectors with at least one pore-containing matrix and causing the cellular source (or a portion thereof) to release all or a portion of the one or more vectors; and

(b) separating or substantially separating the one or more vectors from genomic or chromosomal nucleic acid molecules contained by said cellular source.

In accordance with the invention, the desired low molecular weight nucleic acid molecules or vectors are preferably separated by the matrix based on size separation or size exclusion and thus the matrix can be designed (e.g., varying pore size, matrix material, size and dimension of the matrix, etc.) to separate the desired nucleic acid molecules (e.g., vectors) from unwanted nucleic acid molecules (e.g., chromosomal or genomic nucleic acid molecules) contained by the cellular source. This separation may be facilitated by any means for moving the desired nucleic acid molecules through the matrix including centrifugation, vacuum, gravity, pressure and the like.

According to the invention, the matrix may be any porous matrix that substantially traps or binds (reversibly or irreversibly) high molecular weight nucleic acid molecules but not substantially bind or trap low molecular weight nucleic acid molecules. Suitable materials for preparing the solid matrices used in this aspect of the invention include, but are not limited to, polyester, scintered polyethylene, nitrocellulose, polyolefin, cellulose acetate, cellulose, silica, and the like. This solid matrix may be provided in any convenient format for use in isolation of nucleic acid molecules, for example, as an insert (e.g., a frit or plug or swab or cartridge), as a membrane, as a filter, or as a densely packed porous matrix (e.g., beads or gels). In one aspect, for example, the matrix may be provided as a frit or cartridge or as a membrane suitable for insertion into a tube or column, providing a partitioning of upper and lower chambers of the tube or column by the matrix; such an aspect of the invention is diagramed in Figure 1.

The matrix may also be provided in other convenient forms, such as sheets, frits, plugs, cartridges or inserts suitable to fit multi-well plates typically used in filtration of multiple samples, including, for example, 6-well plates, 12-well plates, 24-well plates, 48-well plates, 96-well plates, 384-well plates, and the like, or suitable to fit into other plate sizes such as 35 mm plates, 60 mm plates, 100 mm plates, 150 mm plates, and the like. In a particularly preferred embodiment, the solid matrix is provided as a frit or insert or cartridge or swab suitable to fit into a microcentrifuge tube, microspin tube or spin cartridges. In one example, the frit/insert/cartridge/swab has a size of 8 mm diameter x 1 cm length. Such tubes are available for example from NNI/Lida Manufacturing, Naperville, IL.

The pores in the separation matrix are typically small enough to prevent passage of large molecular weight (e.g., genomic or chromosomal) nucleic acid molecules, but large enough to permit passage of small molecular weight nucleic acid molecules, and may range from about 0.1 to about 10,000 micrometers in diameter, about 0.1 to about 5,000 micrometers in diameter, about 0.1 to about 1,000 micrometers in diameter, about 1 to about 500 micrometer in diameter, about 10 to about 500 micrometer in diameter, or preferably about 25 to about 400 micrometers in diameter. Larger or smaller pore sizes may also be used, provided the matrix is sufficiently dense so as to provide a "tortuous path" (as that phrase is commonly used by those of ordinary skill in the chromatography arts) preventing direct flow-through of the large molecular weight nucleic acid molecules, but still permitting flow-through of the small molecular weight nucleic acid molecules.

In preferred use, the cellular source is applied onto the matrix, preferably in an aqueous solution, and then is introduced into or on the matrix either by unit gravity incubation or preferably by centrifugation or vacuum. The cellular source will optionally be trapped within or on the matrix in preparation for release of the nucleic acid molecules. Lysis/disruption compositions, physical forces and/or mechanical forces (or combinations thereof) may be used for disrupting the integrity of the cell membrane/cell wall of the cellular source of the nucleic acid molecules. In accordance with the invention, any physical or mechanical forces

(freezing, heating, freeze-thawing, pressure, sonication etc.) may be used separately or in combination with the lysis/disrupting compounds or compositions to release the desired nucleic acid molecules from the cellular source. Preferably, the matrix comprises such lysis/disruption compounds or compositions According to the invention, the lysis/disruption composition or compound may be either applied to the matrix containing the cellular source or preferably may be adsorbed, complexed or associated with (e.g., by ionic, hydrophobic, covalent or non-covalent binding) to the matrix prior to applying the cellular source to the matrix, for example by soaking or saturating the matrix in the disrupting/lysing composition and then allowing the matrix to dry under air, vacuum and/or heat; alternatively, the composition may be applied to the matrix material just prior to its use or prior to the preparation of the matrix plug, frit, insert, membrane, etc. from the matrix material. Any method of pre-treating the matrix results in the formation of a matrix that has been impregnated with a disrupting/lysing composition. Thus, in a preferred aspect, the matrix compises the lysis/disruption compositions or compounds. In this preferred aspect of the invention, contacting of the cellular source and the lysis/disrupting steps of the present methods are thus accomplished concurrently or nearly concurrently, thereby reducing the amount of time and manipulation required for the isolation of the small molecular weight nucleic acid molecules.

In one preferred embodiment, the composition that disrupts the cellular membrane/cell wall integrity that is applied to the matrix, or that is pre-adsorbed onto the matrix, may comprise one or more detergents, such as sodium dodecylsulfate (SDS) or Sarkosyl, Triton X-100, Tween 20, NP-40, N-alkylglucosides, N-alkylmaltosides, glucamides, digitonin, deoxycholate, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) or cetyltrimethyl-ammoniumbromide (CTAB), or Brij 35 at a concentration of about 0.01%-10% (w/v), more preferably about 0.1%-5%, and most preferably about 0.5%; one or more chaeotropic agents such as sodium iodide, sodium perchlorate, guanidine or a salt thereof or urea at a concentration of about 300-1000 mM, more preferably about 500-2000 mM, and most preferably about 1500 mM; one

or more enzymes such as lysozyme, lyticase, zymolyase, neuraminidase, Novozym 234, streptolysin, cellulysin, mutanolysin or lysostaphin at a concentration of about 0.1 to 5 mg/ml; one or more inorganic salts such as sodium chloride, potassium chloride, magnesium chloride, lithium chloride, or praseodymium chloride at a concentration of about 1 mM to 5M; one or more organic solvents such as toluene, phenol, butanol, isopropyl alcohol, isoamyl alcohol, ethanol, an ether (e.g., diethyl ether, dimethyl ether, or ethylmethyl ether), or chloroform at a concentration of 25 to 60% (v/v); or any other compound which disrupts the integrity of (i.e., lyses or causes the formation of pores in) the membrane and/or cell wall of the cellular source of nucleic acid molecules (e.g., polymixin B), or combinations of the foregoing. The compositions may also comprise other components, such as chelating agents (e.g., disodium ethylenediaminetetraacetic acid (Na_2EDTA), EGTA, CDTA, most preferably at a concentration of about 10 mM) and/or one or more ribonucleases (RNase A, T1, T2, and the like) at concentrations ranging from 1 to 400 µg/ml, proteases (Protinase K, Pronase, pepsin, trypsin, papain, subtilisin) at concentrations ranging from 50 to 1000 µg/ml, or any combination of the foregoing. In a particularly preferred embodiment, the composition comprises 0.5% SDS or a combination of triton X-100 and lysozyme. Desired concentrations and combinations of the active ingredients of the lysis/disruption compositions may be readily determined by those skilled in the art.

Once the cellular source of nucleic acid molecules has been contacted with the matrix and the cells ruptured or lysed, the high and low molecular weight nucleic acid molecules contained within the cellular source are released from the cell and the high molecular weight (e.g., genomic or chromosomal) nucleic acid molecules are bound to or trapped within or on the matrix material, while the low molecular weight nucleic acid molecules, such as plasmids, vectors, phagemids, cosmids, and the like, substantially pass through the matrix material without being bound thereby or trapped therein. These low molecular weight nucleic acid molecules may be collected with the flow-through, for example by washing the

matrix with an aqueous solution sufficient to wash or elute the low molecular weight nucleic acid molecules through the matrix, but insufficient to remove the large (genomic or chromosomal) nucleic acid molecules from the matrix to which they are bound or in which they are trapped; such an approach is described in detail in the Example below. If desired, these large molecular weight molecules may be removed from the matrix to which they are bound once being released from the cellular source, by elution with an aqueous solution such as a buffered salt solution, according to methods of nucleic acid chromatography that are well-known in the art.

In accordance with the invention, the desired nucleic acid molecules obtained may be further purified by well known nucleic acid purification or chromatography techniques. In a preferred embodiment, such further purification steps may involve adsorption chromatography, reverse phase ion pair chromatography, ionic exchange chromatography, extraction (e.g., with organic solvents such as phenyl/chloroform), ethanol precipitation, density/gradient centrifugation (CsCl), and the like. Thus, the invention further comprises purifying the desired nucleic acid molecules by any known techniques available in the art. Such additional purification may facilitate removal of unwanted contaminants such as proteins, lipids, nucleotides, oligonucleotides, compounds or compositions which may inhibit further manipulation of the nucleic acid molecule (e.g. by amplification, sequencing, transformation, transfection, nucleic acid synthesis, restriction enzyme digestion, etc.). In any event, such further purification need not take place and thus the nucleic acid molecules obtained by the method of the invention may be manipulated directly by standard molecular biology techniques. In a preferred aspect of the invention, one or more addition further purification resins (e.g., ion exchange resins, and/or absorption resins) are utilized in combination with the separation matrix in accordance with the invention. Such additional purification may be accomplished in separate steps, although in a preferred aspect, the additional purification is accomplished simultaneously or in conjunction with the separation method of the invention. In one aspect, the one or more separation matrices and the one or more nucleic acid

binding resins are associated in series in a fluid channel such that a sample containing the desired nucleic acid molecules may pass from one matrix to another. In this aspect, the separation matrix and binding resin combination may be provided in any format to provide a fluid channel to associate the various matrices in fluid connection such as a column format, a tube format, a well format, a multi-well plate format, etc. In this embodiment, the desired nucleic acid molecules passing through the separation matrix would bind or absorb onto the nucleic acid binding resin. Removal of unwanted materials (such as lipids, proteins, lysis/disruption compositions, and components which may inhibit further manipulation of nucleic acid molecules) are removed with a wash buffer or solution which allows the desired nucleic acid molecule to be retained on the binding resin. An elution buffer or solution for removing the desired nucleic acid molecule from the binding resin may then be used to isolate the purified nucleic acid molecule.

Compositions

In a related aspect, the invention relates to compositions for use in isolating low molecular weight nucleic acid molecules. Compositions according to this aspect of the invention may comprise one or more components or portions, such as:

(a) a cellular source of the desired low molecular weight nucleic acid molecules;

(b) a matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and optionally

(c) at least one compound or composition that disrupts or lysis one or more cells of the cellular source.

Preferred such cellular sources, matrices, and compounds and compositions for use in the compositions of the invention include those described and used in the methods of the present invention. In a preferred composition of the invention, the matrix comprises the compound that disrupts the integrity of the

cellular membrane or cell wall. Such compound is preferably adsorbed onto or complexed with or associated with the matrix, for example by ionic, hydrophobic, non-covalent or covalent attachment of the lysis/disrputing compound or composition to the matrix material. The compositions of the invention are useful in isolating a variety of low molecular weight nucleic acid molecules, particularly those described herein and most particularly plasmids or vectors from bacterial cells.

Kits

In another embodiment, the invention relates to kits for use in isolating low molecular weight nucleic acid molecules. Such kits of the invention may comprise one or more components, which may be contained in or include one or more containers such as boxes, cartons, tubes, microspin tubes, microfuge tubes, spin cartridges, multi-well plates, vials, ampules, bags, and the like. In one such aspect, the kits of the invention may comprise one or more of the compositions of the invention described in detail herein. In another aspect, the kits of the invention may comprise:

(a) at least one matrix which (which is preferably contained in a tube, column, cartridge etc.) substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and

(b) a cell disrupting/lysis composition or compound.

In one such kit, the matrix comprises a cell disrupting/lysing composition or compound which may be may be adsorbed onto or complexed with or associate with the matrix, for example by ionic, hydrophobic, non-covalent or covalent attachment of the composition or compound to the matrix material. In another aspect, the kits comprise additional nucleic acid purification resins (e.g., nucleic acid binding resins), wash buffers, elution buffers etc. Preferred matrix materials, cell lysis/disrupting compositions and compounds, and elution and wash compositions for use in the kits of the invention include those described herein for use in the methods and compositions of the present invention.

The kits of the invention may further comprise one or more additional components or reagents that may be useful in further processing, analysis, or use of the nucleic acid molecules isolated or purified according to the invention, for example components or reagents useful in nucleic acid amplification, sequencing, cloning, transfection, transcription, translation, and the like. Such reagents or components may, for example, include one or more restriction enzymes, one or more polypeptides having nucleic acid polymerase activity, one or more polypeptides having reverse transcriptase activity, one or more cells competent for transformation, one or more transfection reagents (e.g., lipids) and other reagents that will be familiar to one of ordinary skill in the art.

Polypeptides having nucleic acid polymerase activity for use in the kits of the invention may be any polypeptide that can synthesize a nucleic acid molecule from a nucleic acid template, typically in the 5' to 3' direction. The nucleic acid polymerases used in the kits of the present invention may be mesophilic or thermophilic, and are preferably thermophilic. Preferred mesophilic DNA polymerases include T7 DNA polymerase, T5 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III and the like. Preferred thermostable DNA polymerases that may be used in the kits of the invention include *Taq*, *Tne*, *Tma*, *Pfu*, *Tfl*, *Tth*, Stoffel fragment, VENT™ and DEEPVENT™ DNA polymerases, and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent 4,889,818; U.S. Patent 4,965,188; U.S. Patent 5,079,352; U.S. Patent 5,614,365; U.S. Patent 5,374,553; U.S. Patent 5,270,179; U.S. Patent 5,047,342; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; Barnes, W.M., *Gene 112*:29-35 (1992); Lawyer, F.C., *et al.*, *PCR Meth. Appl. 2*:275-287 (1993); Flaman, J.-M, *et al.*, *Nucl. Acids Res. 22*(15):3259-3260 (1994)). For amplification of long nucleic acid molecules (e.g., nucleic acid molecules longer than about 3-5 Kb in length), at least two DNA polymerases (one substantially lacking 3' exonuclease activity and the other having 3' exonuclease activity) are typically used. See U.S. Patent No. 5,436,149; and U.S. Patent No. 5,512,462; Barnes, W.M., *Gene 112*:29-35 (1992), the disclosures of which are incorporated herein in their entireties. Examples of DNA polymerases substantially lacking in

3' exonuclease activity include, but are not limited to, *Taq*, *Tne*(exo⁻), *Tma*(exo⁻), *Pfu*(exo⁻), *Pwo*(exo⁻) and *Tth* DNA polymerases, and mutants, variants and derivatives thereof. RNA polymerases such as T3, T5 and SP6 and mutants, variants and derivatives thereof may also be used in accordance with the invention.

Polypeptides having reverse transcriptase activity for use in the kits of the invention may include any polypeptide having the ability to synthesize an RNA molecule from a DNA template molecule. In one embodiment, the polypeptides having reverse transcriptase activity may be substantially reduced in RNase H activity. Suitable polypeptides having reverse transcriptase activity for use in the kits of the invention include, but are not limited to, M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase or HIV reverse transcriptase. These polypeptides having reverse transcriptase activity may be substantially reduced in RNase H activity; preferred such polypeptides include M-MLV H⁻ reverse transcriptase, RSV H⁻ reverse transcriptase, AMV H⁻ reverse transcriptase, RAV H⁻ reverse transcriptase, MAV H⁻ reverse transcriptase and HIV H⁻ reverse transcriptase. Methods for the production and use of such polypeptides having reverse transcriptase activity, including those which are substantially reduced in RNase H activity, are described in detail in commonly owned, co-pending U.S. Application No. 09/064,057, filed April 22, 1998, the disclosure of which is incorporated herein in its entirety.

Isolated Nucleic Acid Molecules, Vectors, and Host Cells

The invention also relates to isolated nucleic acid molecules that are prepared according to the methods of the invention. According to the invention, the isolated nucleic acid molecules of the invention preferably are low molecular weight nucleic acid molecules. Preferred such low molecular weight nucleic acid molecules that may be isolated according to the present invention include, but are not limited to, plasmids, large molecular weight plasmids (BAC's, PAC's and YAC's), vectors, cDNA molecules or libraries, cosmids, phagemids, organellar nucleic acid molecules (e.g., those isolated from organelles such as mitochondria

or chloroplasts), RNA transcripts, and the like. The nucleic acid molecules may be single stranded or double stranded, circular or linear, supercoiled, and may be comprised of DNA, RNA, or a combination of DNA and RNA. In one preferred embodiment, the isolated nucleic acid molecules of the invention are double-stranded DNA plasmids or vectors (which are optionally supercoiled), particularly those isolated, for example, from bacterial cells.

In a related aspect, the invention provides the ability quickly to screen and evaluate recombinant vectors prepared by recombinant technologies (e.g., by cloning or subcloning). The invention thus may be used to quickly isolate such recombinant vectors, providing a ready source of the recombinant vectors for such evaluation or screening (e.g., by sequencing, restriction digestion, restriction mapping, etc.).

Vectors and recombinant vectors obtained by the invention may be introduced into host cells using any of the techniques for introducing nucleic acid molecules into host cells that are described herein.

The invention also provides recombinant host cells comprising the isolated nucleic acid molecules, vectors or recombinant vectors of the invention. Representative host cells (prokaryotic or eukaryotic) that may be produced according to the invention include, but are not limited to, bacterial cells, yeast cells, plant cells and animal cells. Such suitable host cells are available commercially, for example from Life Technologies, Inc. (Rockville, Maryland), ATCC (Manassas, Virginia), and other commercial sources that will be familiar to one of ordinary skill in the art. Host cells comprising the vectors, recombinant vectors or isolated nucleic acid molecules of the invention may be prepared by inserting the isolated nucleic acid molecules or vectors of the invention into the host cells, using well-known transformation, electroporation or transfection techniques that will be familiar to one of ordinary skill in the art. According to this aspect of the invention, introduction of the isolated nucleic acid molecules into a host cell to produce a host cell comprising the nucleic acid molecules can be effected by any known method of introducing nucleic acid molecules into host cells, including but not limited to calcium phosphate transfection, DEAE-dextran

mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, transformation (e.g., of competent cells particularly *E. coli* cells), infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, "Basic Methods In Molecular Biology" (1986) and Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Appropriate culture media and cultivation conditions for the transformed or transfected host cells are known in the art.

In addition, the invention provides methods for producing a recombinant polypeptide encoded by the isolated nucleic acid molecules of the invention, and polypeptides produced by these methods. According to this aspect of the invention, a recombinant polypeptide may be produced by culturing any of the above recombinant host cells comprising the isolated nucleic acid molecules, recombinant vectors or vectors of the invention, under conditions favoring production of a polypeptide therefrom, and isolation of the polypeptide. Methods for culturing recombinant host cells, and for production and isolation of polypeptides therefrom, are well-known to one of ordinary skill in the art.

Uses of Isolated Nucleic Acid Molecules

The nucleic acid molecules that are isolated by the compositions, methods and kits of the present invention may be further characterized or manipulated, for example by cloning, sequencing, amplification, nucleic acid synthesis, endonuclease digestion and the like.

The isolated nucleic acid molecules of the invention may be used in methods for amplifying and sequencing nucleic acid molecules. Amplification methods which may be used in accordance with the present invention include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA: U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA: U.S. Patent No. 5,409,818; EP 0 329 822). The isolated nucleic acid molecules may also be used in complex PCR-based nucleic acid fingerprinting techniques such as Random Amplified Polymorphic DNA

(RAPD) analysis (Williams, J.G.K., *et al., Nucl. Acids Res. 18(22)*:6531-6535, 1990), Arbitrarily Primed PCR (AP-PCR; Welsh, J., and McClelland, M., *Nucl. Acids Res. 18(24)*:7213-7218, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anollés *et al., Bio/Technology 9*:553-557, 1991), microsatellite PCR or Directed Amplification of Minisatellite-region DNA (DAMD; Heath, D.D., *et al., Nucl. Acids Res. 21(24)*: 5782-5785, 1993), and Amplification Fragment Length Polymorphism (AFLP) analysis (EP 0 534 858; Vos, P., *et al., Nucl. Acids Res. 23(21)*:4407-4414, 1995; Lin, J.J., and Kuo, J., *FOCUS 17(2)*:66-70, 1995). In a particularly preferred aspects, the invention may be used in methods of amplifying or sequencing a nucleic acid molecule comprising one or more polymerase chain reactions (PCRs), such as any of the PCR-based methods described above. Nucleic acid sequencing methods according to this aspect of the invention may comprise both cycle sequencing (sequencing in combination with linear amplification) and standard sequencing reactions, according to methods that are well-known in the art and as described in commonly owned, co-pending U.S. Application No. 08/971,675, filed November 17, 1997, the disclosures of which are incorporated herein in its entirety for its relevant teachings.

Alternatively, nucleic acid molecules isolated according to the present invention may be used for the manufacture of various materials in industrial processes by methods that are well-known in the art. Such materials include, but are not limited to, hybridization probes, therapeutic proteins (dependent upon transcription and translation of the isolated nucleic acid molecules, or the production of synthetic peptides or proteins with amino acid sequences deduced from the nucleotide sequences of the specific nucleic acid molecules), gene therapy vehicles and compositions, molecular weight markers, and the like.

It will be understood by one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are readily apparent and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present

invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

Example 1: Isolation of Plasmid DNA from Bacterial Cells

The aim of this project was to improve the process of preparing plasmid DNA from bacterial cells. Specifically, the objectives were first, to develop a more rapid lysis procedure where there are fewer manipulations and the manipulations are more forgiving, and second, to eliminate a separate centrifugation or filtration step for the removal of precipitated denatured proteins and genomic DNA. According to the present invention, these objectives are accomplished by integration of the lysis and filtration processes into a single operation. The output from this operation is soluble plasmid DNA ready for further purification, if necessary, by matrix chromatography. By the present invention, it is further possible to combine the matrix chromatography step with the lysis and precipitate removal step, to make a single unit operation of the entire plasmid preparation method.

Materials and Methods

All reagents were from Life Technologies, Inc., Rockville, Maryland, unless otherwise noted.

Lysis Matrix. In the initial experiments a Capture Column, a component of the GENERATION DNA Purification Capture Column Kit (Gentra Systems, Minneapolis, MN) was employed according to the manufacturer's instructions. Subsequent experiments used lysis matrices prepared by impregnation of filters with a bacterial lysis solution. Lysis solution, 400 µl of 0.5 % (w/v) sodium dodecyl sulfate, was soaked into a plug or swab tip of bonded polyolefin fibers.

1 cm long x 8mm diameter (Cat. No. 8700-20. Filtrona Richmond. Richmond. VA). The treated swab tips were dried at room temperature for three days. A dried swab tip was placed in a Micro Spin (Cat. No. 8700-20. NNI/Lida Manufacturing. Naperville. IL) for use in cell lysis.

Cell Growth and Lysis. *E. coli* DH10B harboring plasmid pRPA-1 was grown for 16 hrs at 37°C in LB media containing 100µg/ml ampicillin. A 1.5-ml culture sample was centrifuged at 14.000 rpm for 5 min. The supernate was decanted and the pellet was suspended in 200 µl of 50 mM Tris-HCl. pH 8.0. 10 mM EDTA. 166 µg/ml RNase A. The entire suspension was applied to a treated swab tip in a Micro Spin. then incubated at room temperature for 1 min. A 200 µl volume of 10 mM Tris-HCl. pH 8.0. 1 mM EDTA. 166 µg/ml RNase A solution was applied to the same swab tip. then incubated at room temperature for 1 min. The Micro Spin was centrifuged at 14.000 rpm for 1 min and 400 µl of flow-through eluant was collected into a 1.5-ml microcentrifuge tube.

Silica Adsorption Chromatography. Plasmid DNA (200 µl) eluted from the Micro Spin was mixed with 400 µl of 1.5 M Guanidine-HCl. pH 7.5. 12 mM EDTA (GE). The mixture was centrifuged at 14.000 rpm for 2 min to pellet precipitated material. The supernate was transferred to a spin cartridge (CONCERT Rapid Plasmid Miniprep System (Life Technologies)) and centrifuged at 14.000 rpm for 1 min. The remainder of the manufacturer's protocol was followed. Briefly. the spin cartridge was washed twice with 500 µl of wash buffer. then plasmid DNA was eluted with 75 µl of TE buffer pre-warmed to 75°C into a 1.5-ml microcentrifuge tube.

DNA Elution from Lysis Matrix. The Micro Spin from above was treated to elute trapped DNA. To the swap tip was added 200 µl of TE buffer. pH 8.0. The Micro Spin housing the swab tip was incubated in a 100°C water bath for 10 min. Centrifugation of the Micro Spin at 14.000 rpm for 1 min removed the eluant from the swab tip. The volume was collected in a 1.5-ml microcentrifuge tube.

Restriction Digestion of Plasmid DNA. Aliquots (10 µl) of plasmid DNA were incubated for 30 min at 37°C with 10 units of *Hind* III (Life Technologies) according to the manufacturer's instructions.

Results and Discussion

Several experiments were performed to determine whether the GENERATION DNA Purification Capture Column Kit could be used to lyse, capture and release plasmid DNA from bacterial cells. Bacterial cultures harboring plasmid DNAs were successfully lysed when introduced to the Capture Column. However, unlike chromosomal DNA, plasmid DNA was unexpectedly not trapped by the matrix. Rather, plasmid DNA was quantitatively removed from the matrix by simple centrifugation of the lysate volume from the column. Stopping the standard Gentra Systems kit protocol here eliminated the next steps of washing twice, then eluting at 99°C as required for genomic DNA. This truncated protocol achieved the desired advantages over alkaline lysis of faster processing, with fewer, more robust manipulations, and elimination of a separate precipitate removal step. Eluted plasmid DNA in lysis solution was precipitated with alcohol, then resuspended in TE buffer in order to be used in an enzymatic reaction, e.g., a restriction endonuclease digest or *in vitro* amplification.

Since the plasmid DNA eluted from a Capture Column was contaminated with RNA, proteins and other biomolecules, which could interfere with other types of analyses, it was desirable to purify the DNA further with a standard column chromatography system. Adsorption to silica is preferred, since the process is quick and DNA elutes in a ready-to-use form. The composition of the eluant solution from the Capture Column, however, was found to be incompatible with direct plasmid DNA adsorption to a silica matrix. Adsorption to silica requires that the DNA solution be in a specific chemical environment, which includes a high concentration of a chaeotropic agent, such as guanidine•HCl. In order to provide a controlled environment for silica binding after lysis, the cell lysis reagent was simplified, creating a new lysis matrix with 0.5% SDS (described above).

Although less preferred, plasmid DNA may bind directly from the eluant solution to an anion exchange chromatography matrix, e.g., in the CONCERT High Purity Plasmid Miniprep System.

Bacterial cells containing plasmid pRPA-1 were introduced into Suspension Buffer, then applied to an SDS-impregnated swab tip in a Micro Spin. The cell lysis eluant (sample 1) was adjusted to silica adsorption conditions by adding two volumes of GE buffer, forming a precipitate removable by centrifugation. The plasmid-containing supernate was applied to a silica membrane spin cup from the CONCERT Rapid Plasmid Miniprep System. Bound DNA was washed, then eluted in TE buffer (sample 2). To determine which nucleic acids were trapped in the lysis matrix, the Micro Spin was incubated in TE buffer at 100°C for 10 min, then the eluant collected by centrifugation (sample 3).

Aliquots of each sample were analyzed by agarose gel electrophoresis (Figure 2). Plasmid DNA was present in the eluant from the lysis matrix, free of most RNAs and contaminated with a relatively small amount of chromosomal DNA. Adjustment of the chemical environment allowed efficient purification by silica adsorption, as evidenced by the high yield of plasmid and reduction in chromosomal DNA and RNA. Elution of the matrix at elevated temperature showed only fragmented chromosomal DNA, and no plasmid DNA trapped by the matrix. To evaluate the compatibility of the purified DNAs in an enzyme reaction, aliquots of samples 1 and 3 were digested with *Hin*d III. Only DNA cleaned through silica adsorption was cleavable (sample 3). As expected, lysis solution in sample 1 inhibited *Hin*d III activity.

These data demonstrate that plasmid DNA can be isolated from bacterial cells in a simplified process, requiring fewer handling operations than in the standard alkaline lysis. In addition, data have been obtained (not shown) indicating that impregnation of the solid support matrix with a lysis solution containing guanidine permits adsorption of the eluant from the lysis matrix directly onto a silica membrane for further purification.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious

to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for isolating a low molecular weight nucleic acid molecule or population of low molecular weight nucleic acid molecules, comprising:

(a) contacting a cellular source of nucleic acid molecules with at least one pore-containing matrix, which binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap said low molecular weight nucleic acid molecules and causing the cellular source or portion thereof to release all or a portion of the said nucleic acid molecules; and

(b) separating or substantially separating said molecular weight nucleic acid molecules from said high molecular weight nucleic acid molecules.

2. The method of claim 1, wherein said matrix is selected from the group consisting of a polyester matrix, a polyolefin matrix, a sintered polyethylene matrix, a nitrocellulose matrix, a cellulose acetate matrix, a cellulose matrix and a silica matrix.

3. The method of claim 1, wherein said pores in said matrix range from about 1.000 micrometer to about 0.1 micrometers in diameter.

4. The method of claim 1, wherein said pores are from about 500 to about 1 micrometers in diameter.

5. The method of claim 1, wherein said pores are from about 400 to about 25 micrometers in diameter.

6. The method of claim 1, wherein said release of the said nucleic acid molecules are accomplished by a lysis/disruption composition or compound.

7. The method of claim 6, wherein said lysis/disruption composition comprises one or more detergents.

8. The method of claim 6, wherein said lysis/disruption composition comprises one or more chaeotropic agents.

9. The method of claim 8, wherein said chaeotropic agent is guanidine or a salt thereof.

10. The method of claim 6, wherein said lysis/disruption composition comprises one or more enzymes.

11. The method of claim 10, wherein said enzyme is lysozyme, lysostaphin or zymolyase.

12. The method of claim 1, wherein said matrix comprises one or more lysis/disruption compositions or compounds.

13. The method of claim 1, further comprising collecting said low molecular weight nucleic acid molecules.

14. The method of claim 1, wherein said cellular source is a cell selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, an animal cell, a virus and a plant cell.

15. The method of claim 14, wherein said bacterial cell is an *Escherichia coli* cell.

16. The method of claim 1, wherein said low molecular weight nucleic acid molecule is selected from the group consisting of a plasmid, a vector, a

phagemid, a cosmid, a mitochondrial nucleic acid molecule, and a chloroplast nucleic acid molecule.

17. The method of claim 1, wherein said low molecular weight nucleic acid molecule is a DNA molecule.

18. An isolated nucleic acid molecule produced by the method of claim 1.

19. A composition for use in isolating a low molecular weight nucleic acid molecule or a population of nucleic acid molecules, said composition comprising:

(a) a cellular source of said low molecular weight nucleic acid molecules;

(b) a matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and optionally

(c) at least one compound or composition that disrupts or lysis said cellular source.

20. A kit for use in isolating a low molecular weight nucleic acid molecule or a population of nucleic acid molecules, said kit comprising the composition of claim 19.

21. A kit for use in isolating a low molecular weight nucleic acid molecule or a population of nucleic acid molecules, said kit comprising:

(a) at least one matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and

(b) a cell disrupting/lysis composition or compound.

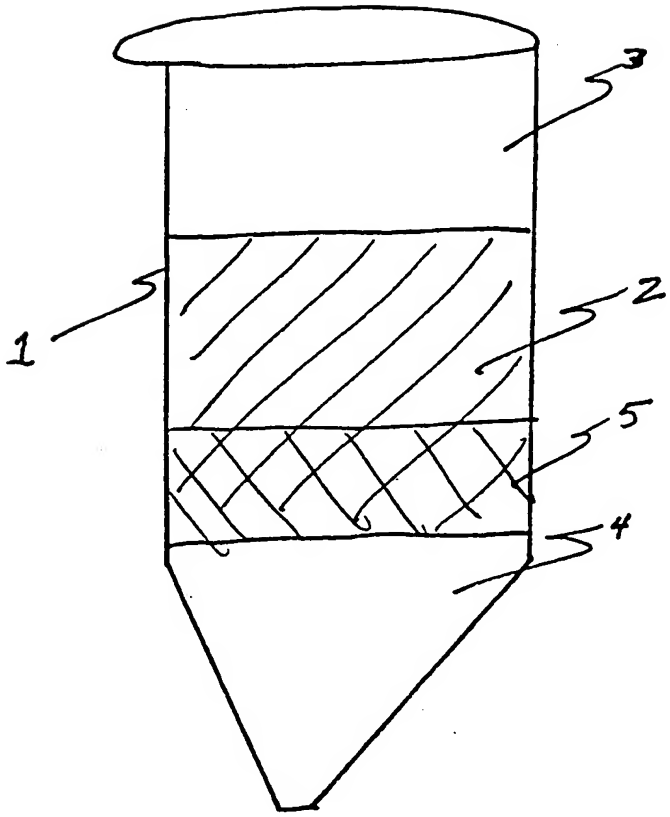

FIGURE 1

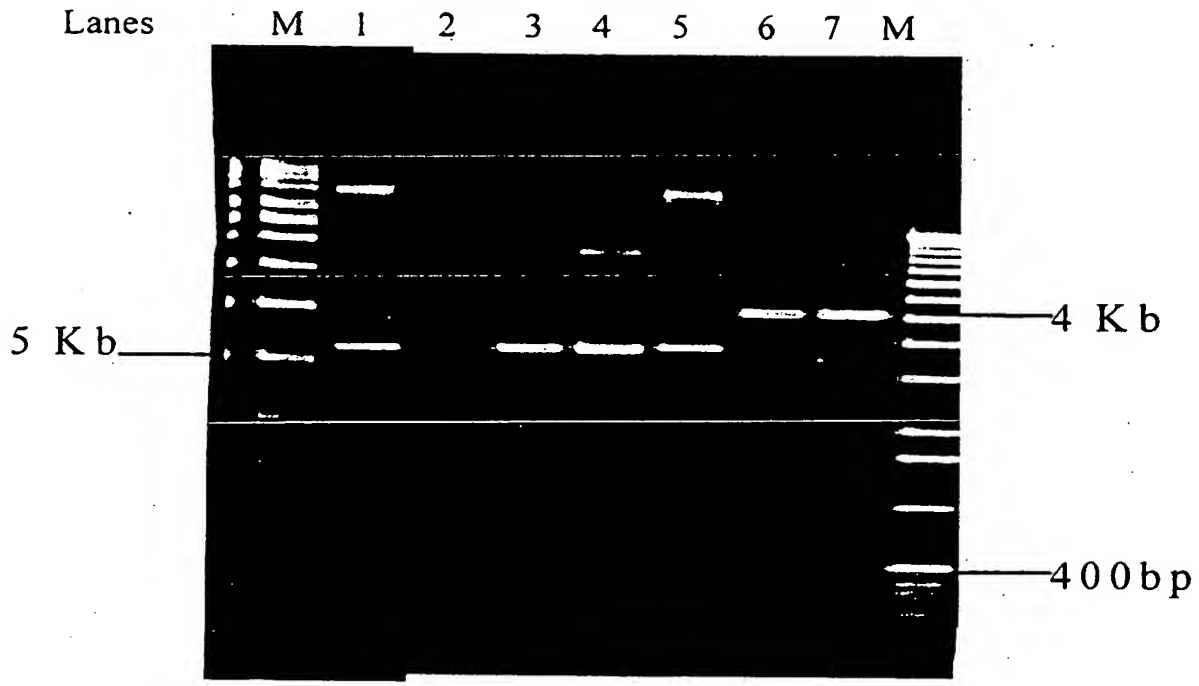

Figure 2

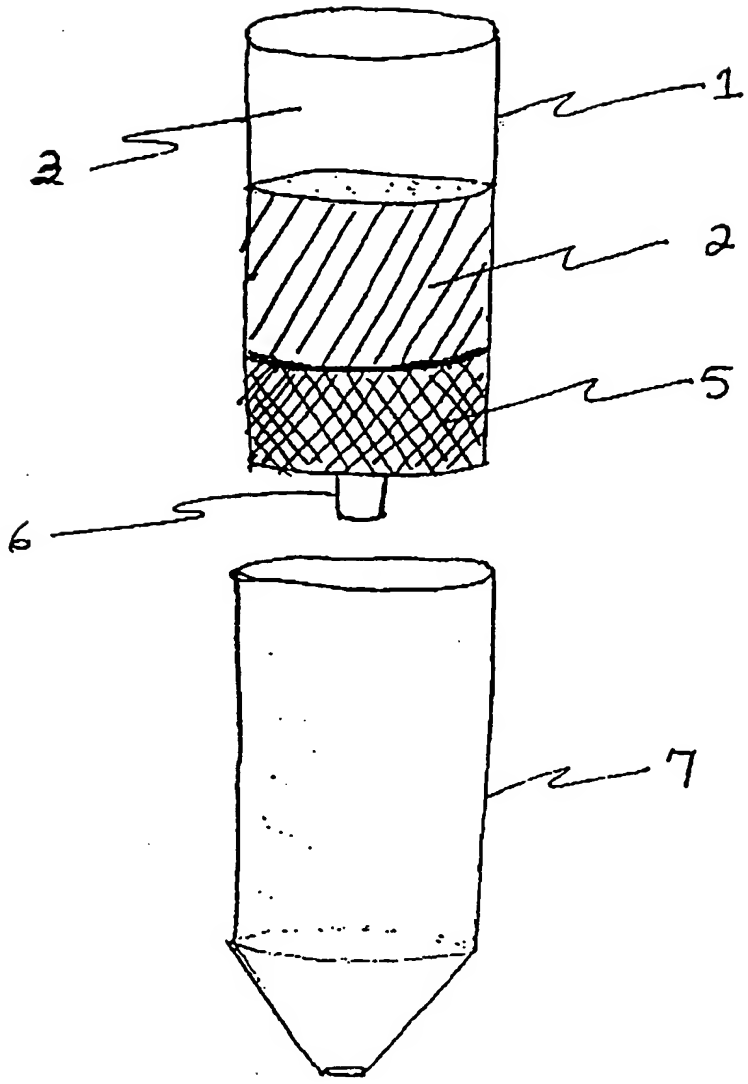

Figure 3

INTERNATIONAL SEARCH REPORT

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US CL :435/270; 536/27
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/270; 536/27, 28

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, BIOSIS, CAPLUS, EMBASE, GENBANK
search terms: purification, isolation, nucleic, matrix, detergent, enzyme, cell, column

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	U S 5,057,426 A (HENCO et al) 15 October 1991, see entire document.	1-19 ------- 20-21
Y	STRATAGENE CATALOG,gene characterization kits, 1988, page 39, see entire document.	20, 21
Y	PROMEGA CATALOG, Wizard Minipreps DNA Purification system. 1993/1994, pages 141-145, see entire document.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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(54) Title: METHODS AND COMPOSITIONS FOR ISOLATION OF NUCLEIC ACID MOLECULES

(57) Abstract

The present invention relates generally to compositions, methods and kits for use in isolating nucleic acid molecules. More specifically, the invention relates to such compositions, methods and kits that are useful in the isolation of nucleic acid molecules from cells (e.g., bacterial cells, animal cells, fungal cells, viruses, yeast cells or plant cells) via lysis and one or more additional isolation steps, such as one or more chromatography steps. In particular, the invention relates to compositions, methods and kits wherein nucleic acid molecules are isolated using an integrated lysis/chromatography matrix, which may comprise one or more supports (e.g., polyolefin, scintered polyethylene, nitrocellulose, polypropylene, polycarbonate, cellulose acetate, silica, and the like) that has been treated with or associated with one or more chemical lysis reagents (e.g., one or more detergents, one or more chaeotropes, one or more enzymes, and the like). The compositions, methods and kits of the invention are suitable for isolating a variety of forms of nucleic acid molecules from cells, including but not limited to plasmids, vectors, DNA, cDNA, RNA, mitochondrial DNA, chloroplast DNA, and the like, any of which may be single–stranded, double–stranded, linear or circular. The compositions, methods and kits of the invention are particularly well–suited for rapid isolation of soluble plasmid or vector DNA from bacterial cells.

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Methods and Compositions for Isolation of Nucleic Acid Molecules

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is in the fields of molecular biology and genetics. The invention relates generally to compositions, methods and kits for use in isolating nucleic acid molecules. More specifically, the invention relates to such compositions, methods and kits that are useful in the isolation of small molecular weight nucleic acid molecules (e.g., vectors, plasmids, and the like) from cells via lysis and one or more additional isolation steps, such as one or more chromatography steps. The compositions, methods and kits of the invention are suitable for isolating a variety of forms of nucleic acid molecules from cells.

Related Art

The most popular method of plasmid DNA isolation from bacterial cells is based on alkaline lysis, followed by batch chromatography. In this lysis procedure (Birnboim, H. and Doly, J. (1979) *Nucleic Acids Res.* 7, 1513) cells are completely ruptured, released proteins and nucleic acids are denatured, then plasmid DNA is preferentially renatured. Precipitated denatured proteins and chromosomal DNA are then separated from soluble plasmid DNA. Typically, plasmid DNA is further purified from residual contaminating proteins, lipids and nucleic acids by selective binding and release from a chromatography matrix. The chromatography matrix, in one instance, is an anion-exchange resin, e.g., Qiagen-tip 20 (Qiagen, and U.S. Patent No. 4,997,932) or CONCERT High Purity Plasmid Miniprep System (Life Technologies, Inc. and U.S. Patent No. 5,843,312). Alternatively, the chromatography matrix is a silica adsorption resin, such as Wizard Minipreps DNA Purification Resin (Promega, and U.S. Patent No. 5,658,548) or CONCERT Rapid Plasmid Miniprep System (Life Technologies,

Inc.). Although effective for the purification of plasmid DNA, several deficiencies in the alkaline lysis method are recognized: first, it represents a relatively long processing time, second, it includes many manual manipulations with at least three different solutions, third, there is a necessary removal of the precipitate prior to chromatography, and fourth, there is a requirement at several steps for careful manipulation by an experienced operator.

Recently, a new type of system was introduced for the purification of genomic, DNA from eukaryotic cells and of chromosomal DNA from Gram-negative bacteria. In the GENERATION DNA Purification Capture Column Kit (Gentra Systems), a purification matrix is used to lyse cells and capture the DNA. Trapped DNA is washed first with buffer, then is released by heating the matrix in Elution Solution for 10 minutes at 99°C, followed by centrifugation. This system combines cell lysis and solid phase chromatography into a single operation, similar to the method of Boom *et al* (U.S. Patent No. 5,234,809). This method is distinct from Boom *et al* in that DNA probably is trapped by the matrix fibers and only released upon denaturation, causing the genomic DNA to be converted into fragmented, single strands. Upon cell lysis in the Boom *et al* method, DNA is adsorbed immediately onto silica particles, which are then washed by a separate filtration operation. DNA is removed from the silica by filtration following application of a low salt buffer at room temperature. The gentle elution conditions maintains native DNA structure, giving the DNA broader utility and is preferred over denatured DNA derived from the GENERATION Kit..

Despite the availability in the art of techniques for isolation of genomic and chromosomal DNA, however, there remains a need in the art for rapid and efficient compositions, methods and kits useful in the isolation of small molecular weight nucleic acid molecules, such as plasmids, vectors, and the like. The present invention provides such compositions, methods and kits.

BRIEF SUMMARY OF THE INVENTION

The present invention relates generally to compositions, methods and kits for use in isolating nucleic acid molecules. More specifically, the invention relates to such compositions, methods and kits that are useful in the isolation of nucleic acid molecules from cells (e.g., bacterial cells, animal cells, fungal cells, yeast cells or plant cells) via lysis and one or more additional isolation steps, such as one or more chromatography steps. In particular, the invention relates to compositions, methods and kits wherein desired nucleic acid molecules are isolated using an integrated lysis/chromatography matrix.

More particularly, the invention relates to methods for isolating nucleic acid molecules, particularly low molecular weight nucleic acid molecules, comprising:

(a) providing one or more cells or a cellular source containing low molecular weight nucleic acid molecules;

(b) contacting the one or more cells or cellular source with at least one pore-containing matrix which binds or traps high molecular weight nucleic acid molecules (e.g., genomic or chromosomal nucleic acid molecules) but does not substantially bind or trap low molecular weight nucleic acid molecules (e.g., vectors, plasmids, etc.);

(c) causing the one or more cells or cellular source (or portion thereof) to lyse or disrupt (e.g., disrupt the integrity of the cell membrane and/or cell wall) such that low and high molecular weight nucleic acid molecules are released from the one or more cells or cellular source; and

(d) collecting the low molecular weight nucleic acid molecules.

In accordance with the invention, the cells may be lysed or disrupted before contacting the cells with the matrix, although cell lysis or disruption preferably takes place after the cells are contacted with the matrix and more preferably at the same time or approximately the same time (e.g., simultaneously

or substantially simultaneously) the cells are contacted with the matrix. In another aspect, the cells are preferably trapped within or on the matrix prior to or during cell lysis or disruption. In yet another aspect, the cells are lysed/disrupted by contacting them with a composition or compound which causes or aids in cell lysis or disruption. although mechanical or physical forces (e.g., pressure, sonication, temperature (heating, freezing), and/or freeze-thawing etc.) may be used in accordance with the invention. In addition, any combination of mechanical forces, physical forces or lysis compositions/compounds may be used to disrupt/lyse the cells. After the one or more cells (or portion thereof) are lysed/disrupted in accordance with the invention, the low molecular weight nucleic acid molecules (or portion thereof) are substantially separated from the high molecular weight nucleic acid molecules. Such separation is preferably accomplished by the matrix binding/trapping the high molecular weight molecules and not substantially trapping/binding the low molecular weight molecules. Such action allows physical separation of such molecules where the smaller molecules of interest are allowed to substantially pass through the matrix while the larger molecules are trapped or bind to the matrix.

According to the invention, the matrix may be any porous matrix that traps or binds high molecular weight and/or does not substantially bind or trap low molecular weight nucleic acid molecules. Such matrix may include but is not limited to a polyester matrix, a polyolefin matrix, a scintered polyethylene matrix, a nitrocellulose matrix, a cellulose acetate matrix, a cellulose matrix, a porous ceramic matrix, a silica matrix, a polysaccharide matrix (Sepharose, agarose, Sephadex, etc.), a polymer matrix (Sephacryl, Trisacryl, Toyopearl, Bio-Gel, etc.) and the like. In a preferred aspect, the matrix is a solid matrix, although the matrix may be a semi-solid matrix. Suitable matrix materials may be obtained commercially, for example from Filtrona Richmond, Inc. (Richmond, Virginia), Bio-Rad (Richmond, California), Gentra Systems (Minneapolis, MN), Tosohaas (Montgomeryville, PA), BioSepra, Inc., (Marlborough, MA), and Porex Technologies Corp. (Fairburn, GA). In a related aspect, the matrix may be prepared in various sizes, shapes, and forms including flat, wafer, cylindrical,

rectangular, beads, gels, square, cartridge, swab tip, plug, frit, membrane and the like, and may also be contained in various containers such as tubes, bottles, vials, ampules, microspin tubes, wells, multi-well plates, bags and the like. In a preferred aspect, the invention involves the use of size separation chromatography and/or filtration to separate or substantially separate low molecular weight nucleic acid molecules (e.g., vectors) from high molecular weight nucleic acid molecules. Thus, any matrix which provides desired size separation (e.g., filters, chromatography supports, etc.) may be used in the invention. One of skill in the art can readily determine the appropriate matrix, pore size of the matrix, size, shape and dimensions of the matrix taking into consideration the type and size of the desired nucleic acid molecules, the cell type or cellular source, the size of the undesired nucleic acid molecules etc. In another aspect, the invention combines such size separation chromatography/filtration with cell lysis/disruption (preferably such lysis/disruption is done when or approximately when the cellular source comes in contact with or after the cellular source is in contact with the chromatography/filtration matrix). The pores or passage ways in the matrix are typically small enough to prevent passage of large nucleic acid molecules but large enough to permit passage of low molecular weight nucleic acid molecules, and may range from about 0.1 to about 10,000 micrometers in diameter, about 0.1 to about 5,000 micrometers in diameter, about 0.1 to about 1,000 micrometers in diameter, about 1 to about 500 micrometer in diameter, about 10 to about 500 micrometer in diameter, or about 25 to about 400 micrometers in diameter.

In one preferred embodiment, the composition or compound that disrupts the cellular membrane or cell wall integrity may comprise one or more detergents, such as sodium dodecylsulfate (SDS) or Sarkosyl, Triton X-100, NP-40, deoxycholate or Brij 35; one or more chaeotropic agents such as sodium iodide, sodium perchlorate or guanidine or a salt thereof; one or more enzymes such as zymolyase, lyticase, lysozyme or lysostaphin; one or more inorganic salts such as sodium chloride, potassium chloride, or lithium chloride; one or more acids and/or bases or buffering agents (e.g., to increase or reduce pH); one or more organic solvents such as toluene, phenol, ethanol, isopropanol, isoamyl alcohol, butanol,

an ether (e.g., diethyl ether, dimethyl ether, or ethylmethyl ether), or chloroform; or any other compound or enzyme which disrupts the integrity of (i.e., lyses or causes the formation of pores in) the cell membrane and/or cell walls (e.g., polymixin B). In another aspect, the composition may comprise one or more compounds or enzymes to degrade, destroy or remove unwanted components or contaminants (e.g., ribonucleases (RNases) to remove or destroy or degrade undesired RNA released from the cellular source and/or proteases (e.g., Proteinase K) to destroy or degrade released proteins). The compositions may also include any combination of the above described components. In one particularly preferred such aspect, the composition may be adsorbed onto or complexed with or associated with the matrix prior to applying the one or more cells or cellular source to the matrix. In a preferred aspect, the composition is dried in or on the matrix. Thus, in a preferred aspect, the matrix comprises a cell lysis/disruption compound or composition. In this aspect, the cell disruption/lysis may occur when or about the same time the cells come into contact with the composition containing matrix. In another aspect, the composition is added after the cells are added to (e.g., bound to or associated with) the matrix. In yet another aspect, the composition is added to the cells prior to adding the cells to the matrix. In this aspect, the composition may be formulated to weaken the cell membrane/cell wall such that the cells will substantially disrupt/lyse when contacted with the matrix. Alternatively, the composition will substantially lyse/disrupt the cells before addition to the matrix.

In accordance with the invention, the nucleic acid molecules of interest (e.g., plasmids, vectors, etc.) may be removed from the matrix by elution with an aqueous solution, such as a buffered salt solution or elution buffer. The unwanted molecules (e.g., chromosomal or genomic DNA) are substantially retained in or on the matrix, thus allowing the desired nucleic acid molecules to be eluted or to be substantially removed from the matrix. Such elution or removal of the desired nucleic acid molecules may be facilitated by centrifugation, gravity, vacuum, pressure, etc., which provides flow of the desired nucleic acid sample from the matrix. The isolated nucleic acid molecules of interest may then be further

purified by standard nucleic acid purification techniques and/or further manipulated by standard molecular biology techniques such as sequencing, amplification, endonuclease digestion (e.g., restriction enzyme digestion), nucleic acid synthesis, transformation, transfection, and the like.

The methods according to the invention are suitable for isolation of low molecular weight nucleic acid molecules from any cell or cellular source, including bacterial cells (particularly *Escherichia coli* cells), yeast cells, fungal cells, animal cells (particularly insect cells, and mammalian cells including human cells, CHO cells, VERO cells, Bowes melanoma cells, HepG2 cells, and the like), and plant cells, any of which may be transformed cells, established cell lines, cancer cells, or normal cells. The methods of the invention are particularly well-suited for isolation of extrachromosomal nucleic acid molecules, including but not limited to plasmids, vectors, phagemids, cosmids, BACs, PACs, YACs, cDNA molecules or cDNA libraries, mitochondrial nucleic acid molecules, and chloroplast nucleic acid molecules, any of which may be single-stranded or double-stranded, linear or circular, supercoiled, and which may be DNA or RNA molecules.

The invention also relates to isolated nucleic acid molecules produced by the methods of the invention, which are preferably low molecular weight nucleic acid molecules, such as the extrachromosomal nucleic acid molecules described herein and particularly plasmids and vectors. The invention also relates to vectors (particularly expression vectors) and host cells comprising these isolated nucleic acid molecules of the invention. The invention also relates to further manipulation of the isolated nucleic acid molecules of the invention by standard molecular biology techniques such as sequencing, nucleic acid synthesis, cloning, amplification, endonuclease digestion (restriction endonuclease digestion), transformation, transfection and the like.

In a related aspect, the invention relates to compositions for use in isolating low molecular weight nucleic acid molecules. Such compositions of the invention preferably comprise one or more components, such as:

(a) a cellular source of the low molecular weight nucleic acid molecules of interest;

(b) a nucleic acid-binding portion comprising at least one pore-containing matrix which binds or traps high molecular weight nucleic acid molecules but not substantially trap or bind low molecular weight nucleic acid molecules; and

(c) a cell disrupting or cell lysis portion comprising at least one compound that disrupts the integrity of the cellular membrane or cell wall when the cellular source comes into contact with said compound.

Preferred cellular sources, solid matrices, and lysis/disrupting compounds for use in the compositions of the invention include those described and used in the methods of the present invention. In a preferred composition of the invention, the compound that disrupts the integrity of the cellular membrane and/or cell wall is adsorbed onto or complexed with or associated with the matrix, for example by ionic, hydrophobic, or covalent or non-covalent attachment of the cell membrane/cell wall disrupting compound to the matrix material. In a preferred aspect, such compound is dried in or on the matrix. The compositions of the invention are useful in isolating a variety of low molecular weight nucleic acid molecules, particularly those described herein and most particularly plasmids and vectors.

The invention also relates to kits for use in isolating low molecular weight nucleic acid molecules, comprising one or more of the components for carrying out the methods of the invention or one or more of the compositions of the invention. Such kits of the invention may comprise one or more components, which may be contained in one or more containers such as boxes, cartons, tubes, vials, ampules, bags, and the like. In one such aspect, the kits of the invention may comprise:

(a) at least one pore-containing matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules (and which preferably traps a cellular source of high and

low molecular weight nucleic acid molecules within or on the matrix): and

(b) a cell disrupting/lysing composition comprising at least one compound that disrupts the integrity of the cellular membrane or cell wall when the cellular source comes into contact with the compound or composition, such that the high and low molecular weight nucleic acid molecules (or portion thereof) are released from the cellular source.

In one such kit, the matrix comprises the cell disrupting/lysing composition or compound. Such cell disrupting/lysing composition or compound may be adsorbed onto or complexed with or associated with the matrix, for example by ionic, hydrophobic, non-covalent or covalent attachment to the matrix material. Such cell disrupting/lysing composition is preferably dried in or on the matrix. Preferred solid matrix materials, cell disrupting/lysing compositions and compounds, and washing and elution compositions for use in the kits of the invention include those described herein for use in the methods of the present invention. In related aspects, the kits of the invention further comprise one or more additional reagents, such as one or more restriction enzymes, one or more polypeptides having nucleic acid polymerase activity (e.g., one or more DNA polymerases which may be thermostable DNA polymerases and/or one or more reverse transcriptases which may be substantially reduced in RNase H activity), one or more cells competent for transformation and/or other transformation (e.g., competent cells) transfection reagents (e.g., cationic lipids) or other components or reagents that may be useful in conjunction with further purification, processing and analysis of the isolated nucleic acid molecules of the invention, for example, components or reagents useful in nucleic acid amplification, sequencing, cloning, transfection, transcription, translation, and the like. Such kits of the invention may also comprise protocols or instructions for carrying out the methods of the invention.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of what is known in the art, the following drawings and description of the invention, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of one aspect of the invention, depicting a thin-walled tube (preferably a microfuge tube of any size) **1** containing a porous, matrix material in the form of a frit or plug or cartridge or swab tip **2** which divides the airspace within the tube into an upper sample application section **3** and a lower sample collection or sample elution section **4**. According to one aspect of the invention, the matrix material **2** may comprise one or more cell disrupting/lysing compounds or compositions. In another aspect, the matrix material may be in the form of beads or a gel or other semi-solid matrix in which case the matrix is preferably encased or associated with a solid support material to maintain the upper sample-application section **3** and the lower sample collection section **4**. Preferably, the matrix material (solid or semi-solid) is in the form of a cartridge or plug or swab tip which can be easily removed from the tube **1** to facilitate sample collection. In another aspect, one or more additional matrices or resins may be included in the upper sample application section **3** and/or in the sample-collection section **4**, to further facilitate isolation or purification of the desired nucleic acid molecules. For example, well known nucleic acid binding matrices (such as silica adsorption resins, anion-exchange resins, reverse phase resins, and/or affinity resins) may be included below a size separation matrix of the invention to further remove or substantially remove undesired components including proteins, lipids, lysis/disruption compositions used to lyse/disrupt the cellular source, solvents, detergents, etc. Alternatively, matrices (such as cation-exchange resins, reverse phase resins, and/or hydrophobic interaction resins) which bind undesired such components but which do not substantially bind the desired nucleic acids may be used. In another embodiment, combinations of such nucleic acid binding matrices and contaminant binding matrices may be used. The optional nucleic acid binding

resin and/or contaminant binding resin **5** is shown. Such additional matrices are preferably in cartridge or plug or swab tip form. In another aspect, the sample-collection section **4** may contain an opening or access port (which may be closed if desired) to collect samples without the need to remove the matrix or matrices. In one example, where a size separation matrix and a nucleic acid binding matrix are provided, the desired nucleic acid molecules pass through the size separation matrix and bind to the binding matrix. Then be applied to remove unwanted materials through the access port or opening within the sample-collection section **4**. If desired, prior to the addition of the wash buffers, the seze separation matrix may be removed from the tube **1**. The desired isolated nucleic acid molecules may then be removed from the access port/opening when an elution buffer is applied. Alternatively, the removal of desired nucleic acid molecules is accomplished by removal of the matrix or matrices to access the sample-collection section **4**.

Figure 2 is a photograph of an ethidium bromide-stained 1% agarose gel, analyzing samples of nucleic acid molecules at various stages of isolation by the methods of the invention. Lane 1: 10µl of flow through from swab tip (Sample 1); lane 2: 10µl of trapped DNA released from swab tip (Sample 2); lane 3: 10µl of eluant from Concert Rapid spin cartridge (Sample 3); lane 4: Purified plasmid pRPA-1; lane 5: 10µl of Sample 1 digested with 10 units of *Hin*d III; lane 6: 10µl of Sample 3 digested with 10 units of *Hin*d III; lane 7: Purified pRPA-1 digested with 10 units of *Hin*d III; M: Molecular weight markers -- Supercoiled DNA Ladder (left) and 1 Kb DNA Ladder (right).

Figure 3 is a diagram of one aspect of the invention, depicting a thin-walled tube or column (preferably microspin or spin cartridges of any size) **1** containing a size separation matrix (for separating large nucleic acid molecules from small nuclei acid molecules) **2** and a second matrix material **5** for further purifying small molecular weight nucleic acid molecules. Preferably, the matrix material **5** is a nucleic acid binding matrix or a contaminant binding matrix, or combinations thereof. The size separation matrix **2** and the preferred nucleic acid binding matrix **5** may be separated by a space within the tube or column **1**.

although such matrices are preferably in close proximity and preferably separated by a frit or other material. The tube of column 1 contains a sample application section 3 and an opening or access port 6 (which may be closed if desired) to collect the sample. An optional collection tube, well or container 7 is provided for collecting samples passing through the opening or access port 6. In a preferred aspect, the size separation matrix 2 comprises a cell lysis/disruption compound or composition. In the application of such preferred embodiment, a sample containing a cellular source of large and small molecular weight nucleic acid molecules are applied to the sample application section 3 preferably to the upper surface of the matrix 2. The cell lysis/disruption composition or compound causes release of the low and/or high molecular weight nucleic acid molecules which separate according to size in the size separation matrix 2, allowing low molecular weight nucleic acid molecules to pass through the matrix 2, while a substantial portion of the large molecular weight nucleic acid molecules are retained in or on the matrix 2. Small molecular weight nucleic acid molecules passing through the size separation matrix 2 bind to the nucleic acid binding matrix 5. The size separation matrix 2 may optionally be removed from the column or tube 1 (before or after washing) to minimize large molecular weight nucleic acid molecules from passing through the size separation matrix 2 during subsequent washing and elution steps. Washing buffers or solutions may then be applied to remove unwanted materials. An elution buffer or solution may then be applied to elute the desired low molecular weight nucleic acid molecules from the nucleic acid binding matrix and through the opening or access port 6. During washing, the collection tube 7 (containing the unwanted materials) can be replaced with a second or new collection tube 7 to collect the desired nucleic acid molecules upon elution.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions, methods, and kits that may be used in isolating nucleic acid molecules from a nucleic acid-containing sample. It will be readily appreciated by those skilled in the art that, in accordance with the

present invention, any cell, tissues, organs, populations of cells, etc. can be used as a nucleic acid source. Preferably, such nucleic acid sources are used to isolate any given plasmid, vector, or other extragenomic or extrachromosomal structure. In a particular aspect, a population of different vectors (for example, a cDNA library or genomic library contained by the cell population or culture) is used as a nucleic acid source. Thus, the invnetion allows isolation of such population of vectors/cDNA library/genomic library.

In the description that follows, a number of terms used in the fields of molecular biology and recombinant DNA technology are utilized extensively. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Low molecular weight nucleic acid molecule. As used herein, the phrase "low molecular weight nucleic acid molecule" refers to any nucleic acid molecule or population of nucleic acid molecules which is smaller in size or molecular mass than a genomic nucleic acid molecule such as a chromosome from a given cell or cellular source. Low molecular weight nucleic acid molecules according to the invention typically are no larger than about 500 kilobases in size, and are preferably about 1 to about 300, more preferably about 1 to about 200, more preferably about 1 to about 100, still more preferably about 1 to about 50, still more preferably about 1 to about 25 and most preferably about 1 to about 15 kilobases in size. In contrast, the term "high molecular weight nucleic acid molecule" is used to mean any nucleic acid molecule or population of nucleic acid molecules that is larger than about 500 kilobases in size, particularly genomic nucleic acid molecules or chromosomes which may range in size from about 1 to about 15,000 megabases. Examples of low molecular weight nucleic acid molecules include, but are not limited to, plasmids, large molecular weight plasmids (BAC's, PAC's, YAC's), vectors, phagemids, cosmids, mitochondrial nucleic acid molecules, chloroplast nucleic acid molecules, cDNA molecules or cDNA libraries, amplification fragments (e.g., PCR generated nucleic acid molecules) and fragments of nucleic acid molecules regardless of the means by

which such fragments are generated. Such fragments may be generated by enzymatic digestion (e.g., endonuclease digestion for example with type I and/or II restriction enzymes), mechanical forces (shearing) and the like. In particular, fragments of genomic or chromosomal nucleic acid molecules which are smaller in size than the complete genomic or chromosomal nucleic acid molecule from which they are derived are included in this definition.

Amplification. As used herein, "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a nucleic acid (e.g., DNA) molecule or primer thereby forming a new nucleic acid molecule complementary to the nucleic acid template. The formed nucleic acid molecule and its template can be used as templates to synthesize additional nucleic acid molecules. As used herein, one amplification reaction may consist of many rounds of nucleic acid synthesis. Amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a nucleic acid molecule.

Host. Any prokaryotic or eukaryotic cell that is the recipient of a replicable expression vector or cloning vector. The terms "host" or "host cell" may be used interchangeably herein. For examples of such hosts, see Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Preferred prokaryotic hosts include, but are not limited to, bacteria of the genus *Escherichia* (e.g., *E. coli*), *Bacillus*, *Staphylococcus*, *Agrobacter* (e.g., *A. tumefaciens*), *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, *Caryophanon*, etc. The most preferred prokaryotic host is *E. coli*. Bacterial hosts of particular interest in the present invention include *E. coli* strains K12, DH10B, DH5α and HB101. Preferred eukaryotic hosts include, but are not limited to, fungi, fish cells, yeast cells, plant cells and animal cells. Particularly preferred animal cells are insect cells such as *Drosophila* cells, *Spodoptera* Sf9, Sf21 cells and *Trichoplusa* High-Five cells; nematode cells such

as *C. elegans* cells; and mammalian cells such as COS cells, CHO cells, VERO cells, 293 cells, PERC6 cells, BHK cells and human cells. In accordance with the invention, a host or host cell may serve as the cellular source for the desired nucleic acid molecule to be isolated.

Vector. A vector is a nucleic acid molecule (preferably DNA) capable of replicating autonomously in a host cell. Such vectors may also be characterized by having a small number of endonuclease restriction sites at which such sequences may be cut without loss of an essential biological function and into which nucleic acid molecules may be spliced to bring about its replication and cloning. Examples include plasmids, autonomously replicating sequences (ARS), centromeres, cosmids and phagemids. Vectors can further provide primer sites, e.g., for PCR, transcriptional and/or translational initiation and/or regulation sites, recombinational signals, recombination sites (see, for example, co-pending U.S. application serial no. 08/663,002 filed June 7, 1996 and U.S. application serial no. 60/065,930 filed October 24, 1997), replicons, etc. The vector can further contain one or more selectable markers suitable for use in the identification of cells transformed or transfected with the vector, such as kanamycin, tetracycline, amplicillin, etc.

In accordance with the invention, any vector may be used. In particular, vectors known in the art and those commercially available (and variants or derivatives thereof) may be used in accordance with the invention. Such vectors may be obtained from, for example, Vector Laboratories Inc., InVitrogen, Promega, Novagen, NEB, Clontech, Boehringer Mannheim, Pharmacia, EpiCenter, OriGenes Technologies Inc., Stratagene, Perkin Elmer, Pharmingen, Life Technologies, Inc., and Research Genetics. Such vectors may be used for cloning or subcloning nucleic acid molecules of interest and therefore recombinant vectors containing inserts, nucleic acid fragments or genes may also be isolated in accordance with the invention. General classes of vectors of particular interest include prokaryotic and/or eukaryotic cloning vectors, expression vectors, fusion vectors, two-hybrid or reverse two-hybrid vectors, shuttle vectors for use in different hosts, mutagenesis vectors, transcription vectors, vectors for receiving

large inserts (yeast artifical chromosomes (YAC's), bacterial artificial chromosomes (BAC's) and P1 artifical chromosomes (PAC's)) and the like. Other vectors of interest include viral origin vectors (M13 vectors, bacterial phage λ vectors, baculovirus vectors, adenovirus vectors, and retrovirus vectors), high, low and adjustable copy number vectors, vectors which have compatible replicons for use in combination in a single host (e.g., pACYC184 and pBR322) and eukaryotic episomal replication vectors (e.g., pCDM8). The vectors contemplated by the invention include vectors containing inserted or additional nucleic acid fragments or sequences (e.g., recombinant vectors) as well as derivatives or variants of any of the vectors described herein.

Expression vectors useful in accordance with the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids or bacteriophages, and vectors derived from combinations thereof, such as cosmids and phagemids, and will preferably include at least one selectable marker (such as a tetracycline or ampicillin resistance genes) and one or more promoters such as the phage lambda P_L promoter, and/or the *E. coli lac*, *trp* and *tac* promoters. Other suitable promoters will be known to the skilled artisan.

Among vectors preferred for use in the present invention include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; pcDNA3 available from Invitrogen; pGEX, pTrxfus, pTrc99a, pET-5, pET-9, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia; and pSPORT1, pSPORT2 and pSV·SPORT1, available from Life Technologies, Inc. Other suitable vectors will be readily apparent to the skilled artisan.

Plasmid. As used herein, the term plasmid means an extrachromosomal genetic element, typically less than about 25 kilobases (kb) in size and more typically about 15 kb to about 2 kb in size.

Isolated. As used herein, the term "isolated" (as in "isolated nucleic acid molecule") means that the isolated material, component, or composition has been

at least partially purified away from other materials, contaminants, and the like which are not part of the material, component, or composition that has been isolated. For example, an "isolated low molecular weight nucleic acid molecule" is a nucleic acid molecule that has been treated in such a way as to remove at least some of the other nucleic acid molecules (e.g., large nucleic acid molecules) with which it may be associated in the cell, tissue, organ or organism. In particular, the term "isolated low molecular weight nucleic acid molecule" or "isolated vector" refers to a low molecular weight nucleic acid molecule preparation or vector preparation which contains no more than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, and 7%, preferably no more than 5%, 2.5%, and 2%, and most preferably less than 1%, 0.5%, and 0.1% (percentages by weight) of high molecular weight nucleic acid molecules (e.g., chromosomal/genomic DNA). As one of ordinary skill will appreciate, however, a solution comprising an isolated nucleic acid molecule may comprise one or more buffer salts and/or a solvents, e.g., water or an organic solvent such as acetone, ethanol, methanol, and the like, and yet the nucleic acid molecule may still be considered an "isolated" nucleic acid molecule with respect to its starting materials.

Cell disrupting or cell lysing compound or composition. As used herein, "cell disrupting" or "cell lysing" refers to a composition or a component of a composition that effects lysis, rupture, or poration of the cells, tissues, or organisms used as the source of the nucleic acid molecules to be isolated, such that the nucleic acid molecules (or portion thereof) that are contained in the cell, tissue, or organism source are released from the cell, tissue, or organism. According to the invention, the cells, tissues, or organisms need not be completely lysed, ruptured or porated, and all of the nucleic acid molecules contained in the source cells, tissues or organisms need not be released therefrom. Preferably, a cell disrupting or cell lysis compound or composition at least 25%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or more of the total nucleic acid molecules, particularly the total low molecular weight nucleic acid molecules (such as vectors, plasmids, and the like) that are contained in the cell, tissue, or organism.

Other terms used in the fields of recombinant DNA technology and molecular and cell biology as used herein will be generally understood by one of ordinary skill in the applicable arts.

Sources of DNA

The methods, compositions and kits of the invention are suitable for isolation of low molecular weight nucleic acid molecules from any cellular source, including a variety of cells, tissues, organs or organisms, which may be natural or which may be obtained through any number of commercial sources (including American Type Culture Collection (ATCC), Rockville, Maryland; Jackson Laboratories, Bar Harbor, Maine; Cell Systems, Inc., Kirkland, Washington; Advanced Tissue Sciences, La Jolla, California). Cells that may be used as cellular nucleic acid sources may be prokaryotic (bacterial, including members of the genera *Escherichia* (particularly *E. coli*), *Serratia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Chlamydia*, *Neisseria*, *Treponema*, *Mycoplasma*, *Borrelia*, *Bordetella*, *Legionella*, *Pseudomonas*, *Mycobacterium*, *Helicobacter*, *Agrobacterium*, *Collectotrichum*, *Rhizobium*, and *Streptomyces*) or eukaryotic (including fungi or yeasts, plants, protozoans and other parasites, and animals including humans and other mammals). Any virus may also be used as a cellular source of nucleic acid molecules in accordance with the invention. Also suitable for use as sources of low molecular weight nucleic acid molecules are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus. These cells, tissues and organs may be normal, transformed, or established cell lines, or they may be pathological such as those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS) or parasites), in genetic or biochemical pathologies (e.g., cystic fibrosis, hemophilia, Alzheimer's disease, schizophrenia, muscular dystrophy or multiple sclerosis), or in cancers and cancerous processes. The methods, compositions and kits of the invention are particularly well-suited for isolation of

extrachromosomal nucleic acid molecules, including but not limited to plasmids, vectors, phagemids, cosmids, cDNA molecules, mitochondrial nucleic acid molecules, and chloroplast nucleic acid molecules, any of which may be single-stranded or double-stranded, linear or circular, supercoiled, and which may be DNA or RNA molecules. In a particularly preferred aspect, the methods of the invention are useful in the isolation of plasmid or vector DNA from bacterial cells. Other cells, tissues, viruses, organs and organisms that will be familiar to one of ordinary skill in the art may also be used as sources of nucleic acid molecules for the preparation of isolated nucleic acid molecules according to the present invention.

Methods

In one aspect, the invention relates to methods for isolating nucleic acid molecules, particularly small molecular weight nucleic acid molecules such as plasmids, vectors, organellar nucleic acid molecules, and the like. Methods according to this aspect of the invention may comprise one or more steps which result in the isolation of one or more nucleic acid molecules or populations of nucleic acid molecules (e.g., a cDNA library) from the natural environment in which the nucleic acid molecules are found.

In one preferred such aspect, the methods of the invention may comprise:

(a) contacting a cellular source of nucleic acid molecules, with at least one pore-containing matrix which binds or traps high molecular weight nucleic acid molecules (e.g., genomic or chromosomal nucleic acid molecules) but does not substantially bind or trap low molecular weight nucleic acid molecules and causing the cellular source (or portion thereof) to release all or a portion of the desired low molecular weight nucleic acid molecules; and

(b) separating or substantially separating the low molecular weight nucleic acid molecules from the high molecular weight nucleic acid molecules.

More specifically, the invention relates to a method for obtaining one or more vectors comprising:

(a) contacting a cellular source of one or more vectors with at least one pore-containing matrix and causing the cellular source (or a portion thereof) to release all or a portion of the one or more vectors; and

(b) separating or substantially separating the one or more vectors from genomic or chromosomal nucleic acid molecules contained by said cellular source.

In accordance with the invention, the desired low molecular weight nucleic acid molecules or vectors are preferably separated by the matrix based on size separation or size exclusion and thus the matrix can be designed (e.g., varying pore size, matrix material, size and dimension of the matrix, etc.) to separate the desired nucleic acid molecules (e.g., vectors) from unwanted nucleic acid molecules (e.g., chromosomal or genomic nucleic acid molecules) contained by the cellular source. This separation may be facilitated by any means for moving the desired nucleic acid molecules through the matrix including centrifugation, vacuum, gravity, pressure and the like.

According to the invention, the matrix may be any porous matrix that substantially traps or binds (reversibly or irreversibly) high molecular weight nucleic acid molecules but not substantially bind or trap low molecular weight nucleic acid molecules. Suitable materials for preparing the solid matrices used in this aspect of the invention include, but are not limited to, polyester, scintered polyethylene, nitrocellulose, polyolefin, cellulose acetate, cellulose, silica, and the like. This solid matrix may be provided in any convenient format for use in isolation of nucleic acid molecules, for example, as an insert (e.g., a frit or plug or swab or cartridge), as a membrane, as a filter, or as a densely packed porous matrix (e.g., beads or gels). In one aspect, for example, the matrix may be provided as a frit or cartridge or as a membrane suitable for insertion into a tube or column, providing a partitioning of upper and lower chambers of the tube or column by the matrix; such an aspect of the invention is diagramed in Figure 1.

The matrix may also be provided in other convenient forms, such as sheets, frits, plugs, cartridges or inserts suitable to fit multi-well plates typically used in filtration of multiple samples, including, for example, 6-well plates, 12-well plates, 24-well plates, 48-well plates, 96-well plates, 384-well plates, and the like, or suitable to fit into other plate sizes such as 35 mm plates, 60 mm plates, 100 mm plates, 150 mm plates, and the like. In a particularly preferred embodiment, the solid matrix is provided as a frit or insert or cartridge or swab suitable to fit into a microcentrifuge tube, microspin tube or spin cartridges. In one example, the frit/insert/cartridge/swab has a size of 8 mm diameter x 1 cm length. Such tubes are available for example from NNI/Lida Manufacturing, Naperville, IL.

The pores in the separation matrix are typically small enough to prevent passage of large molecular weight (e.g., genomic or chromosomal) nucleic acid molecules, but large enough to permit passage of small molecular weight nucleic acid molecules, and may range from about 0.1 to about 10,000 micrometers in diameter, about 0.1 to about 5,000 micrometers in diameter, about 0.1 to about 1,000 micrometers in diameter, about 1 to about 500 micrometer in diameter, about 10 to about 500 micrometer in diameter, or preferably about 25 to about 400 micrometers in diameter. Larger or smaller pore sizes may also be used, provided the matrix is sufficiently dense so as to provide a "tortuous path" (as that phrase is commonly used by those of ordinary skill in the chromatography arts) preventing direct flow-through of the large molecular weight nucleic acid molecules, but still permitting flow-through of the small molecular weight nucleic acid molecules.

In preferred use, the cellular source is applied onto the matrix, preferably in an aqueous solution, and then is introduced into or on the matrix either by unit gravity incubation or preferably by centrifugation or vacuum. The cellular source will optionally be trapped within or on the matrix in preparation for release of the nucleic acid molecules. Lysis/disruption compositions, physical forces and/or mechanical forces (or combinations thereof) may be used for disrupting the integrity of the cell membrane/cell wall of the cellular source of the nucleic acid molecules. In accordance with the invention, any physical or mechanical forces

(freezing, heating, freeze-thawing, pressure, sonication etc.) may be used separately or in combination with the lysis/disrupting compounds or compositions to release the desired nucleic acid molecules from the cellular source. Preferably, the matrix comprises such lysis/disruption compounds or compositions According to the invention, the lysis/disruption composition or compound may be either applied to the matrix containing the cellular source or preferably may be adsorbed, complexed or associated with (e.g., by ionic, hydrophobic, covalent or non-covalent binding) to the matrix prior to applying the cellular source to the matrix, for example by soaking or saturating the matrix in the disrupting/lysing composition and then allowing the matrix to dry under air, vacuum and/or heat; alternatively, the composition may be applied to the matrix material just prior to its use or prior to the preparation of the matrix plug, frit, insert, membrane, etc. from the matrix material. Any method of pre-treating the matrix results in the formation of a matrix that has been impregnated with a disrupting/lysing composition. Thus, in a preferred aspect, the matrix compises the lysis/disruption compositions or compounds. In this preferred aspect of the invention, contacting of the cellular source and the lysis/disrupting steps of the present methods are thus accomplished concurrently or nearly concurrently, thereby reducing the amount of time and manipulation required for the isolation of the small molecular weight nucleic acid molecules.

In one preferred embodiment, the composition that disrupts the cellular membrane/cell wall integrity that is applied to the matrix, or that is pre-adsorbed onto the matrix, may comprise one or more detergents, such as sodium dodecylsulfate (SDS) or Sarkosyl, Triton X-100, Tween 20, NP-40, N-alkylglucosides, N-alkylmaltosides, glucamides, digitonin, deoxycholate, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) or cetyltrimethyl-ammoniumbromide (CTAB), or Brij 35 at a concentration of about 0.01%-10% (w/v), more preferably about 0.1%-5%, and most preferably about 0.5%; one or more chaeotropic agents such as sodium iodide, sodium perchlorate, guanidine or a salt thereof or urea at a concentration of about 300-1000 mM, more preferably about 500-2000 mM, and most preferably about 1500 mM; one

or more enzymes such as lysozyme, lyticase, zymolyase, neuraminidase, Novozym 234, streptolysin, cellulysin, mutanolysin or lysostaphin at a concentration of about 0.1 to 5 mg/ml; one or more inorganic salts such as sodium chloride, potassium chloride, magnesium chloride, lithium chloride, or praseodymium chloride at a concentration of about 1 mM to 5M; one or more organic solvents such as toluene, phenol, butanol, isopropyl alcohol, isoamyl alcohol, ethanol, an ether (e.g., diethyl ether, dimethyl ether, or ethylmethyl ether), or chloroform at a concentration of 25 to 60% (v/v); or any other compound which disrupts the integrity of (i.e., lyses or causes the formation of pores in) the membrane and/or cell wall of the cellular source of nucleic acid molecules (e.g., polymixin B), or combinations of the foregoing. The compositions may also comprise other components, such as chelating agents (e.g., disodium ethylenediaminetetraacetic acid (Na_2EDTA), EGTA, CDTA, most preferably at a concentration of about 10 mM) and/or one or more ribonucleases (RNase A, T1, T2, and the like) at concentrations ranging from 1 to 400 μg/ml, proteases (Protinase K, Pronase, pepsin, trypsin, papain, subtilisin) at concentrations ranging from 50 to 1000 μg/ml, or any combination of the foregoing. In a particularly preferred embodiment, the composition comprises 0.5% SDS or a combination of triton X-100 and lysozyme. Desired concentrations and combinations of the active ingredients of the lysis/disruption compositions may be readily determined by those skilled in the art.

Once the cellular source of nucleic acid molecules has been contacted with the matrix and the cells ruptured or lysed, the high and low molecular weight nucleic acid molecules contained within the cellular source are released from the cell and the high molecular weight (e.g., genomic or chromosomal) nucleic acid molecules are bound to or trapped within or on the matrix material, while the low molecular weight nucleic acid molecules, such as plasmids, vectors, phagemids, cosmids, and the like, substantially pass through the matrix material without being bound thereby or trapped therein. These low molecular weight nucleic acid molecules may be collected with the flow-through, for example by washing the

matrix with an aqueous solution sufficient to wash or elute the low molecular weight nucleic acid molecules through the matrix, but insufficient to remove the large (genomic or chromosomal) nucleic acid molecules from the matrix to which they are bound or in which they are trapped: such an approach is described in detail in the Example below. If desired, these large molecular weight molecules may be removed from the matrix to which they are bound once being released from the cellular source, by elution with an aqueous solution such as a buffered salt solution, according to methods of nucleic acid chromatography that are well-known in the art.

In accordance with the invention, the desired nucleic acid molecules obtained may be further purified by well known nucleic acid purification or chromatography techniques. In a preferred embodiment, such further purification steps may involve adsorption chromatography, reverse phase ion pair chromatography, ionic exchange chromatography, extraction (e.g., with organic solvents such as phenyl/chloroform), ethanol precipitation, density/gradient centrifugation (CsCl), and the like. Thus, the invention further comprises purifying the desired nucleic acid molecules by any known techniques available in the art. Such additional purification may facilitate removal of unwanted contaminants such as proteins, lipids, nucleotides, oligonucleotides, compounds or compositions which may inhibit further manipulation of the nucleic acid molecule (e.g. by amplification, sequencing, transformation, transfection, nucleic acid synthesis, restriction enzyme digestion, etc.). In any event, such further purification need not take place and thus the nucleic acid molecules obtained by the method of the invention may be manipulated directly by standard molecular biology techniques. In a preferred aspect of the invention, one or more addition further purification resins (e.g., ion exchange resins, and/or absorption resins) are utilized in combination with the separation matrix in accordance with the invention. Such additional purification may be accomplished in separate steps, although in a preferred aspect, the additional purification is accomplished simultaneously or in conjunction with the separation method of the invention. In one aspect, the one or more separation matrices and the one or more nucleic acid

binding resins are associated in series in a fluid channel such that a sample containing the desired nucleic acid molecules may pass from one matrix to another. In this aspect, the separation matrix and binding resin combination may be provided in any format to provide a fluid channel to associate the various matrices in fluid connection such as a column format, a tube format, a well format, a multi-well plate format, etc. In this embodiment, the desired nucleic acid molecules passing through the separation matrix would bind or absorb onto the nucleic acid binding resin. Removal of unwanted materials (such as lipids, proteins, lysis/disruption compositions, and components which may inhibit further manipulation of nucleic acid molecules) are removed with a wash buffer or solution which allows the desired nucleic acid molecule to be retained on the binding resin. An elution buffer or solution for removing the desired nucleic acid molecule from the binding resin may then be used to isolate the purified nucleic acid molecule.

Compositions

In a related aspect, the invention relates to compositions for use in isolating low molecular weight nucleic acid molecules. Compositions according to this aspect of the invention may comprise one or more components or portions, such as:

(a) a cellular source of the desired low molecular weight nucleic acid molecules:

(b) a matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules: and optionally

(c) at least one compound or composition that disrupts or lysis one or more cells of the cellular source.

Preferred such cellular sources, matrices, and compounds and compositions for use in the compositions of the invention include those described and used in the methods of the present invention. In a preferred composition of the invention, the matrix comprises the compound that disrupts the integrity of the

cellular membrane or cell wall. Such compound is preferably adsorbed onto or complexed with or associated with the matrix, for example by ionic, hydrophobic, non-covalent or covalent attachment of the lysis/disrputing compound or composition to the matrix material. The compositions of the invention are useful in isolating a variety of low molecular weight nucleic acid molecules, particularly those described herein and most particularly plasmids or vectors from bacterial cells.

Kits

In another embodiment, the invention relates to kits for use in isolating low molecular weight nucleic acid molecules. Such kits of the invention may comprise one or more components, which may be contained in or include one or more containers such as boxes, cartons, tubes, microspin tubes, microfuge tubes, spin cartridges, multi-well plates, vials, ampules, bags, and the like. In one such aspect, the kits of the invention may comprise one or more of the compositions of the invention described in detail herein. In another aspect, the kits of the invention may comprise:

(a) at least one matrix which (which is preferably contained in a tube, column, cartridge etc.) substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and

(b) a cell disrupting/lysis composition or compound.

In one such kit, the matrix comprises a cell disrupting/lysing composition or compound which may be may be adsorbed onto or complexed with or associate with the matrix, for example by ionic, hydrophobic, non-covalent or covalent attachment of the composition or compound to the matrix material. In another aspect, the kits comprise additional nucleic acid purification resins (e.g., nucleic acid binding resins), wash buffers, elution buffers etc. Preferred matrix materials, cell lysis/disrupting compositions and compounds, and elution and wash compositions for use in the kits of the invention include those described herein for use in the methods and compositions of the present invention.

The kits of the invention may further comprise one or more additional components or reagents that may be useful in further processing, analysis, or use of the nucleic acid molecules isolated or purified according to the invention, for example components or reagents useful in nucleic acid amplification, sequencing, cloning, transfection, transcription, translation, and the like. Such reagents or components may, for example, include one or more restriction enzymes, one or more polypeptides having nucleic acid polymerase activity, one or more polypeptides having reverse transcriptase activity, one or more cells competent for transformation, one or more transfection reagents (e.g., lipids) and other reagents that will be familiar to one of ordinary skill in the art.

Polypeptides having nucleic acid polymerase activity for use in the kits of the invention may be any polypeptide that can synthesize a nucleic acid molecule from a nucleic acid template, typically in the 5' to 3' direction. The nucleic acid polymerases used in the kits of the present invention may be mesophilic or thermophilic, and are preferably thermophilic. Preferred mesophilic DNA polymerases include T7 DNA polymerase, T5 DNA polymerase, Klenow fragment DNA polymerase, DNA polymerase III and the like. Preferred thermostable DNA polymerases that may be used in the kits of the invention include *Taq*, *Tne*, *Tma*, *Pfu*, *Tfl*, *Tth*, Stoffel fragment, VENT™ and DEEPVENT™ DNA polymerases, and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent 4,889,818; U.S. Patent 4,965,188; U.S. Patent 5,079,352; U.S. Patent 5,614,365; U.S. Patent 5,374,553; U.S. Patent 5,270,179; U.S. Patent 5,047,342; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; Barnes, W.M., *Gene 112*:29-35 (1992); Lawyer, F.C., *et al.*, *PCR Meth. Appl. 2*:275-287 (1993); Flaman, J.-M. *et al.*, *Nucl. Acids Res. 22*(15):3259-3260 (1994)). For amplification of long nucleic acid molecules (e.g., nucleic acid molecules longer than about 3-5 Kb in length), at least two DNA polymerases (one substantially lacking 3' exonuclease activity and the other having 3' exonuclease activity) are typically used. See U.S. Patent No. 5,436,149; and U.S. Patent No. 5,512,462; Barnes, W.M., *Gene 112*:29-35 (1992), the disclosures of which are incorporated herein in their entireties. Examples of DNA polymerases substantially lacking in

3' exonuclease activity include, but are not limited to, *Taq*, *Tne*(exo⁻), *Tma*(exo⁻), *Pfu*(exo⁻), *Pwo*(exo⁻) and *Tth* DNA polymerases, and mutants, variants and derivatives thereof. RNA polymerases such as T3, T5 and SP6 and mutants, variants and derivatives thereof may also be used in accordance with the invention.

Polypeptides having reverse transcriptase activity for use in the kits of the invention may include any polypeptide having the ability to synthesize an RNA molecule from a DNA template molecule. In one embodiment, the polypeptides having reverse transcriptase activity may be substantially reduced in RNase H activity. Suitable polypeptides having reverse transcriptase activity for use in the kits of the invention include, but are not limited to, M-MLV reverse transcriptase, RSV reverse transcriptase, AMV reverse transcriptase, RAV reverse transcriptase, MAV reverse transcriptase or HIV reverse transcriptase. These polypeptides having reverse transcriptase activity may be substantially reduced in RNase H activity; preferred such polypeptides include M-MLV H⁻ reverse transcriptase, RSV H⁻ reverse transcriptase, AMV H⁻ reverse transcriptase, RAV H⁻ reverse transcriptase, MAV H⁻ reverse transcriptase and HIV H⁻ reverse transcriptase. Methods for the production and use of such polypeptides having reverse transcriptase activity, including those which are substantially reduced in RNase H activity, are described in detail in commonly owned, co-pending U.S. Application No. 09/064,057, filed April 22, 1998, the disclosure of which is incorporated herein in its entirety.

Isolated Nucleic Acid Molecules, Vectors, and Host Cells

The invention also relates to isolated nucleic acid molecules that are prepared according to the methods of the invention. According to the invention, the isolated nucleic acid molecules of the invention preferably are low molecular weight nucleic acid molecules. Preferred such low molecular weight nucleic acid molecules that may be isolated according to the present invention include, but are not limited to, plasmids, large molecular weight plasmids (BAC's, PAC's and YAC's), vectors, cDNA molecules or libraries, cosmids, phagemids, organellar nucleic acid molecules (e.g., those isolated from organelles such as mitochondria

or chloroplasts), RNA transcripts, and the like. The nucleic acid molecules may be single stranded or double stranded, circular or linear, supercoiled, and may be comprised of DNA, RNA, or a combination of DNA and RNA. In one preferred embodiment, the isolated nucleic acid molecules of the invention are double-stranded DNA plasmids or vectors (which are optionally supercoiled), particularly those isolated, for example, from bacterial cells.

In a related aspect, the invention provides the ability quickly to screen and evaluate recombinant vectors prepared by recombinant technologies (e.g., by cloning or subcloning). The invention thus may be used to quickly isolate such recombinant vectors, providing a ready source of the recombinant vectors for such evaluation or screening (e.g., by sequencing, restriction digestion, restriction mapping, etc.).

Vectors and recombinant vectors obtained by the invention may be introduced into host cells using any of the techniques for introducing nucleic acid molecules into host cells that are described herein.

The invention also provides recombinant host cells comprising the isolated nucleic acid molecules, vectors or recombinant vectors of the invention. Representative host cells (prokaryotic or eukaryotic) that may be produced according to the invention include, but are not limited to, bacterial cells, yeast cells, plant cells and animal cells. Such suitable host cells are available commercially, for example from Life Technologies, Inc. (Rockville, Maryland), ATCC (Manassas, Virginia), and other commercial sources that will be familiar to one of ordinary skill in the art. Host cells comprising the vectors, recombinant vectors or isolated nucleic acid molecules of the invention may be prepared by inserting the isolated nucleic acid molecules or vectors of the invention into the host cells, using well-known transformation, electroporation or transfection techniques that will be familiar to one of ordinary skill in the art. According to this aspect of the invention, introduction of the isolated nucleic acid molecules into a host cell to produce a host cell comprising the nucleic acid molecules can be effected by any known method of introducing nucleic acid molecules into host cells, including but not limited to calcium phosphate transfection, DEAE-dextran

mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, transformation (e.g., of competent cells particularly *E. coli* cells), infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, "Basic Methods In Molecular Biology" (1986) and Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). Appropriate culture media and cultivation conditions for the transformed or transfected host cells are known in the art.

In addition, the invention provides methods for producing a recombinant polypeptide encoded by the isolated nucleic acid molecules of the invention, and polypeptides produced by these methods. According to this aspect of the invention, a recombinant polypeptide may be produced by culturing any of the above recombinant host cells comprising the isolated nucleic acid molecules, recombinant vectors or vectors of the invention, under conditions favoring production of a polypeptide therefrom, and isolation of the polypeptide. Methods for culturing recombinant host cells, and for production and isolation of polypeptides therefrom, are well-known to one of ordinary skill in the art.

Uses of Isolated Nucleic Acid Molecules

The nucleic acid molecules that are isolated by the compositions, methods and kits of the present invention may be further characterized or manipulated, for example, by cloning, sequencing, amplification, nucleic acid synthesis, endonuclease digestion and the like.

The isolated nucleic acid molecules of the invention may be used in methods for amplifying and sequencing nucleic acid molecules. Amplification methods which may be used in accordance with the present invention include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA: U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822). The isolated nucleic acid molecules may also be used in complex PCR-based nucleic acid fingerprinting techniques such as Random Amplified Polymorphic DNA

(RAPD) analysis (Williams, J.G.K., *et al.*, *Nucl. Acids Res. 18(22)*:6531-6535, 1990), Arbitrarily Primed PCR (AP-PCR; Welsh, J., and McClelland, M., *Nucl. Acids Res. 18(24)*:7213-7218, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anollés *et al.*, *Bio/Technology 9*:553-557, 1991), microsatellite PCR or Directed Amplification of Minisatellite-region DNA (DAMD; Heath, D.D., *et al.*, *Nucl. Acids Res. 21(24)*: 5782-5785, 1993), and Amplification Fragment Length Polymorphism (AFLP) analysis (EP 0 534 858; Vos, P., *et al.*, *Nucl. Acids Res. 23(21)*:4407-4414, 1995; Lin, J.J., and Kuo, J., *FOCUS 17(2)*:66-70, 1995). In a particularly preferred aspects, the invention may be used in methods of amplifying or sequencing a nucleic acid molecule comprising one or more polymerase chain reactions (PCRs), such as any of the PCR-based methods described above. Nucleic acid sequencing methods according to this aspect of the invention may comprise both cycle sequencing (sequencing in combination with linear amplification) and standard sequencing reactions, according to methods that are well-known in the art and as described in commonly owned, co-pending U.S. Application No. 08/971,675, filed November 17, 1997, the disclosures of which are incorporated herein in its entirety for its relevant teachings.

Alternatively, nucleic acid molecules isolated according to the present invention may be used for the manufacture of various materials in industrial processes by methods that are well-known in the art. Such materials include, but are not limited to, hybridization probes, therapeutic proteins (dependent upon transcription and translation of the isolated nucleic acid molecules, or the production of synthetic peptides or proteins with amino acid sequences deduced from the nucleotide sequences of the specific nucleic acid molecules), gene therapy vehicles and compositions, molecular weight markers, and the like.

It will be understood by one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are readily apparent and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present

invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

Example 1: Isolation of Plasmid DNA from Bacterial Cells

The aim of this project was to improve the process of preparing plasmid DNA from bacterial cells. Specifically, the objectives were first, to develop a more rapid lysis procedure where there are fewer manipulations and the manipulations are more forgiving, and second, to eliminate a separate centrifugation or filtration step for the removal of precipitated denatured proteins and genomic DNA. According to the present invention, these objectives are accomplished by integration of the lysis and filtration processes into a single operation. The output from this operation is soluble plasmid DNA ready for further purification, if necessary, by matrix chromatography. By the present invention, it is further possible to combine the matrix chromatography step with the lysis and precipitate removal step, to make a single unit operation of the entire plasmid preparation method.

Materials and Methods.

All reagents were from Life Technologies, Inc., Rockville, Maryland, unless otherwise noted.

Lysis Matrix. In the initial experiments a Capture Column, a component of the GENERATION DNA Purification Capture Column Kit (Gentra Systems, Minneapolis, MN) was employed according to the manufacturer's instructions. Subsequent experiments used lysis matrices prepared by impregnation of filters with a bacterial lysis solution. Lysis solution, 400 µl of 0.5 % (w/v) sodium dodecyl sulfate, was soaked into a plug or swab tip of bonded polyolefin fibers.

1 cm long x 8mm diameter (Cat. No. 8700-20. Filtrona Richmond. Richmond. VA). The treated swab tips were dried at room temperature for three days. A dried swab tip was placed in a Micro Spin (Cat. No. 8700-20. NNI/Lida Manufacturing. Naperville. IL) for use in cell lysis.

Cell Growth and Lysis. *E. coli* DH10B harboring plasmid pRPA-1 was grown for 16 hrs at 37°C in LB media containing 100µg/ml ampicillin. A 1.5-ml culture sample was centrifuged at 14.000 rpm for 5 min. The supernate was decanted and the pellet was suspended in 200 µl of 50 mM Tris-HCl. pH 8.0, 10 mM EDTA. 166 µg/ml RNase A. The entire suspension was applied to a treated swab tip in a Micro Spin. then incubated at room temperature for 1 min. A 200 µl volume of 10 mM Tris-HCl. pH 8.0. 1 mM EDTA. 166 µg/ml RNase A solution was applied to the same swab tip. then incubated at room temperature for 1 min. The Micro Spin was centrifuged at 14.000 rpm for 1 min and 400 µl of flow-through eluant was collected into a 1.5-ml microcentrifuge tube.

Silica Adsorption Chromatography. Plasmid DNA (200 µl) eluted from the Micro Spin was mixed with 400 µl of 1.5 M Guanidine-HCl. pH 7.5. 12 mM EDTA (GE). The mixture was centrifuged at 14.000 rpm for 2 min to pellet precipitated material. The supernate was transferred to a spin cartridge (CONCERT Rapid Plasmid Miniprep System (Life Technologies)) and centrifuged at 14.000 rpm for 1 min. The remainder of the manufacturer's protocol was followed. Briefly. the spin cartridge was washed twice with 500 µl of wash buffer. then plasmid DNA was eluted with 75 µl of TE buffer pre-warmed to 75°C into a 1.5-ml microcentrifuge tube.

DNA Elution from Lysis Matrix. The Micro Spin from above was treated to elute trapped DNA. To the swap tip was added 200 µl of TE buffer. pH 8.0. The Micro Spin housing the swab tip was incubated in a 100°C water bath for 10 min. Centrifugation of the Micro Spin at 14.000 rpm for 1 min removed the eluant from the swab tip. The volume was collected in a 1.5-ml microcentrifuge tube.

Restriction Digestion of Plasmid DNA. Aliquots (10 µl) of plasmid DNA were incubated for 30 min at 37°C with 10 units of *Hind* III (Life Technologies) according to the manufacturer's instructions.

Results and Discussion

Several experiments were performed to determine whether the GENERATION DNA Purification Capture Column Kit could be used to lyse, capture and release plasmid DNA from bacterial cells. Bacterial cultures harboring plasmid DNAs were successfully lysed when introduced to the Capture Column. However, unlike chromosomal DNA, plasmid DNA was unexpectedly not trapped by the matrix. Rather, plasmid DNA was quantitatively removed from the matrix by simple centrifugation of the lysate volume from the column. Stopping the standard Gentra Systems kit protocol here eliminated the next steps of washing twice, then eluting at 99°C as required for genomic DNA. This truncated protocol achieved the desired advantages over alkaline lysis of faster processing, with fewer, more robust manipulations, and elimination of a separate precipitate removal step. Eluted plasmid DNA in lysis solution was precipitated with alcohol, then resuspended in TE buffer in order to be used in an enzymatic reaction, e.g., a restriction endonuclease digest or *in vitro* amplification.

Since the plasmid DNA eluted from a Capture Column was contaminated with RNA, proteins and other biomolecules, which could interfere with other types of analyses, it was desirable to purify the DNA further with a standard column chromatography system. Adsorption to silica is preferred, since the process is quick and DNA elutes in a ready-to-use form. The composition of the eluant solution from the Capture Column, however, was found to be incompatible with direct plasmid DNA adsorption to a silica matrix. Adsorption to silica requires that the DNA solution be in a specific chemical environment, which includes a high concentration of a chaeotropic agent, such as guanidine•HCl. In order to provide a controlled environment for silica binding after lysis, the cell lysis reagent was simplified, creating a new lysis matrix with 0.5% SDS (described above).

Although less preferred, plasmid DNA may bind directly from the eluant solution to an anion exchange chromatography matrix, e.g., in the CONCERT High Purity Plasmid Miniprep System.

Bacterial cells containing plasmid pRPA-1 were introduced into Suspension Buffer, then applied to an SDS-impregnated swab tip in a Micro Spin. The cell lysis eluant (sample 1) was adjusted to silica adsorption conditions by adding two volumes of GE buffer, forming a precipitate removable by centrifugation. The plasmid-containing supernate was applied to a silica membrane spin cup from the CONCERT Rapid Plasmid Miniprep System. Bound DNA was washed, then eluted in TE buffer (sample 2). To determine which nucleic acids were trapped in the lysis matrix, the Micro Spin was incubated in TE buffer at 100°C for 10 min, then the eluant collected by centrifugation (sample 3).

Aliquots of each sample were analyzed by agarose gel electrophoresis (Figure 2). Plasmid DNA was present in the eluant from the lysis matrix, free of most RNAs and contaminated with a relatively small amount of chromosomal DNA. Adjustment of the chemical environment allowed efficient purification by silica adsorption, as evidenced by the high yield of plasmid and reduction in chromosomal DNA and RNA. Elution of the matrix at elevated temperature showed only fragmented chromosomal DNA, and no plasmid DNA trapped by the matrix. To evaluate the compatibility of the purified DNAs in an enzyme reaction, aliquots of samples 1 and 3 were digested with *Hind* III. Only DNA cleaned through silica adsorption was cleavable (sample 3). As expected, lysis solution in sample 1 inhibited *Hind* III activity.

These data demonstrate that plasmid DNA can be isolated from bacterial cells in a simplified process, requiring fewer handling operations than in the standard alkaline lysis. In addition, data have been obtained (not shown) indicating that impregnation of the solid support matrix with a lysis solution containing guanidine permits adsorption of the eluant from the lysis matrix directly onto a silica membrane for further purification.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious

to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for isolating a low molecular weight nucleic acid molecule or population of low molecular weight nucleic acid molecules. comprising:

(a) contacting a cellular source of nucleic acid molecules with at least one pore-containing matrix. which binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap said low molecular weight nucleic acid molecules and causing the cellular source or portion thereof to release all or a portion of the said nucleic acid molecules: and

(b) separating or substantially separating said molecular weight nucleic acid molecules from said high molecular weight nucleic acid molecules.

2. The method of claim 1. wherein said matrix is selected from the group consisting of a polyester matrix. a polyolefin matrix. a sintered polyethylene matrix. a nitrocellulose matrix. a cellulose acetate matrix. a cellulose matrix and a silica matrix.

3. The method of claim 1. wherein said pores in said matrix range from about 1.000 micrometer to about 0.1 micrometers in diameter.

4. The method of claim 1. wherein said pores are from about 500 to about 1 micrometers in diameter.

5. The method of claim 1. wherein said pores are from about 400 to about 25 micrometers in diameter.

6. The method of claim 1. wherein said release of the said nucleic acid molecules are accomplished by a lysis/disruption composition or compound.

7. The method of claim 6, wherein said lysis/disruption composition comprises one or more detergents.

8. The method of claim 6, wherein said lysis/disruption composition comprises one or more chaeotropic agents.

9. The method of claim 8, wherein said chaeotropic agent is guanidine or a salt thereof.

10. The method of claim 6, wherein said lysis/disruption composition comprises one or more enzymes.

11. The method of claim 10, wherein said enzyme is lysozyme, lysostaphin or zymolyase.

12. The method of claim 1, wherein said matrix comprises one or more lysis/disruption compositions or compounds.

13. The method of claim 1, further comprising collecting said low molecular weight nucleic acid molecules.

14. The method of claim 1, wherein said cellular source is a cell selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, an animal cell, a virus and a plant cell.

15. The method of claim 14, wherein said bacterial cell is an *Escherichia coli* cell.

16. The method of claim 1, wherein said low molecular weight nucleic acid molecule is selected from the group consisting of a plasmid, a vector, a

phagemid, a cosmid, a mitochondrial nucleic acid molecule, and a chloroplast nucleic acid molecule.

17. The method of claim 1, wherein said low molecular weight nucleic acid molecule is a DNA molecule.

18. An isolated nucleic acid molecule produced by the method of claim 1.

19. A composition for use in isolating a low molecular weight nucleic acid molecule or a population of nucleic acid molecules, said composition comprising:

(a) a cellular source of said low molecular weight nucleic acid molecules;

(b) a matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and optionally

(c) at least one compound or composition that disrupts or lysis said cellular source.

20. A kit for use in isolating a low molecular weight nucleic acid molecule or a population of nucleic acid molecules, said kit comprising the composition of claim 19.

21. A kit for use in isolating a low molecular weight nucleic acid molecule or a population of nucleic acid molecules, said kit comprising:

(a) at least one matrix which substantially binds or traps high molecular weight nucleic acid molecules but does not substantially bind or trap low molecular weight nucleic acid molecules; and

(b) a cell disrupting/lysis composition or compound.

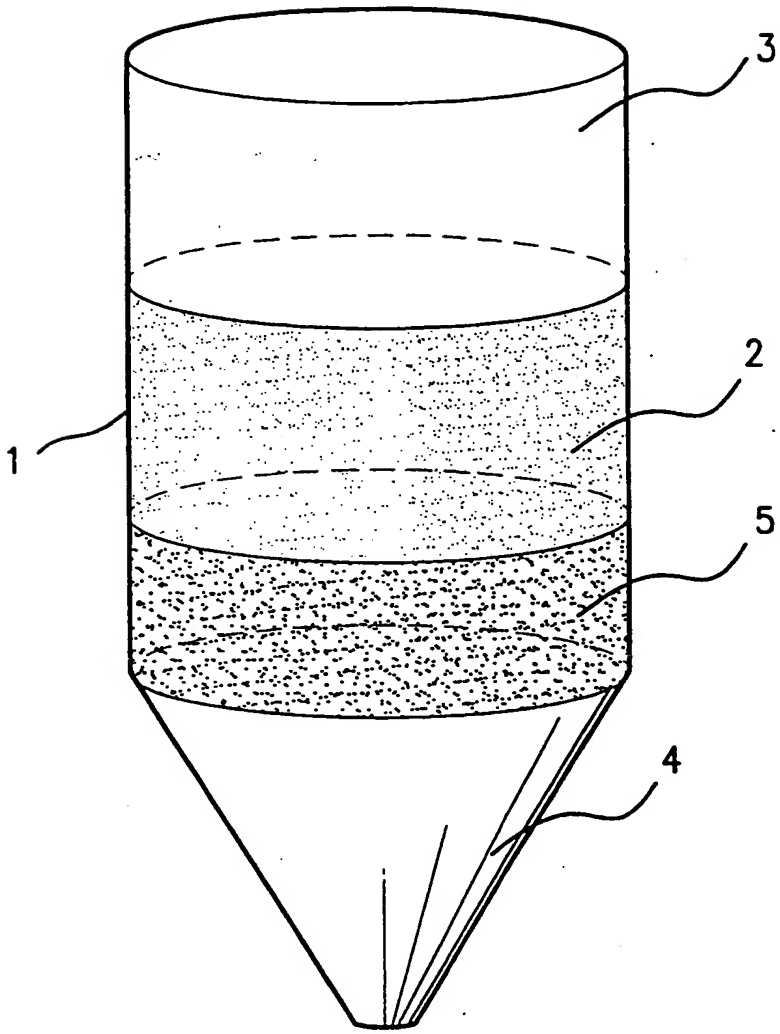
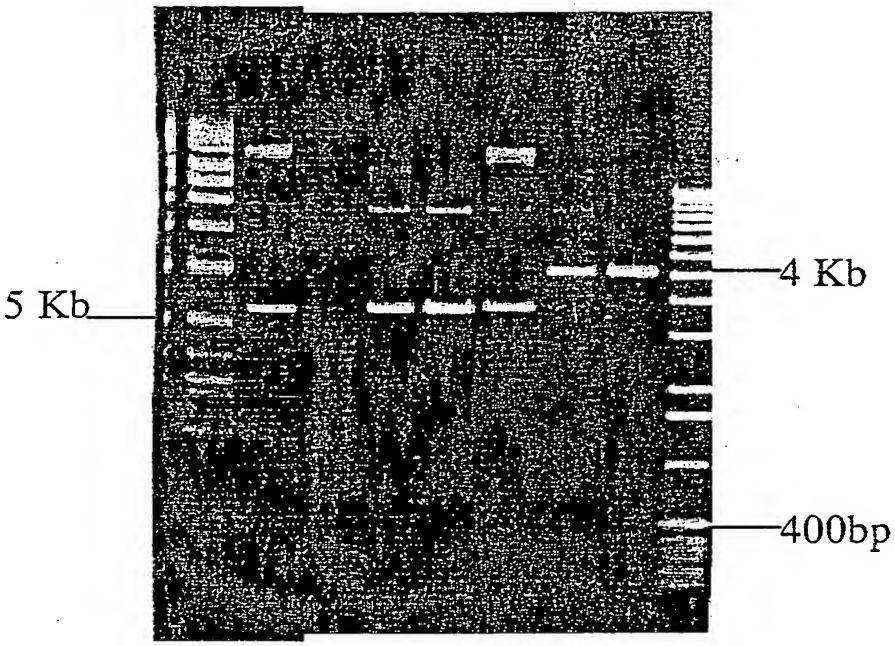


FIG.1

FIGURE 2



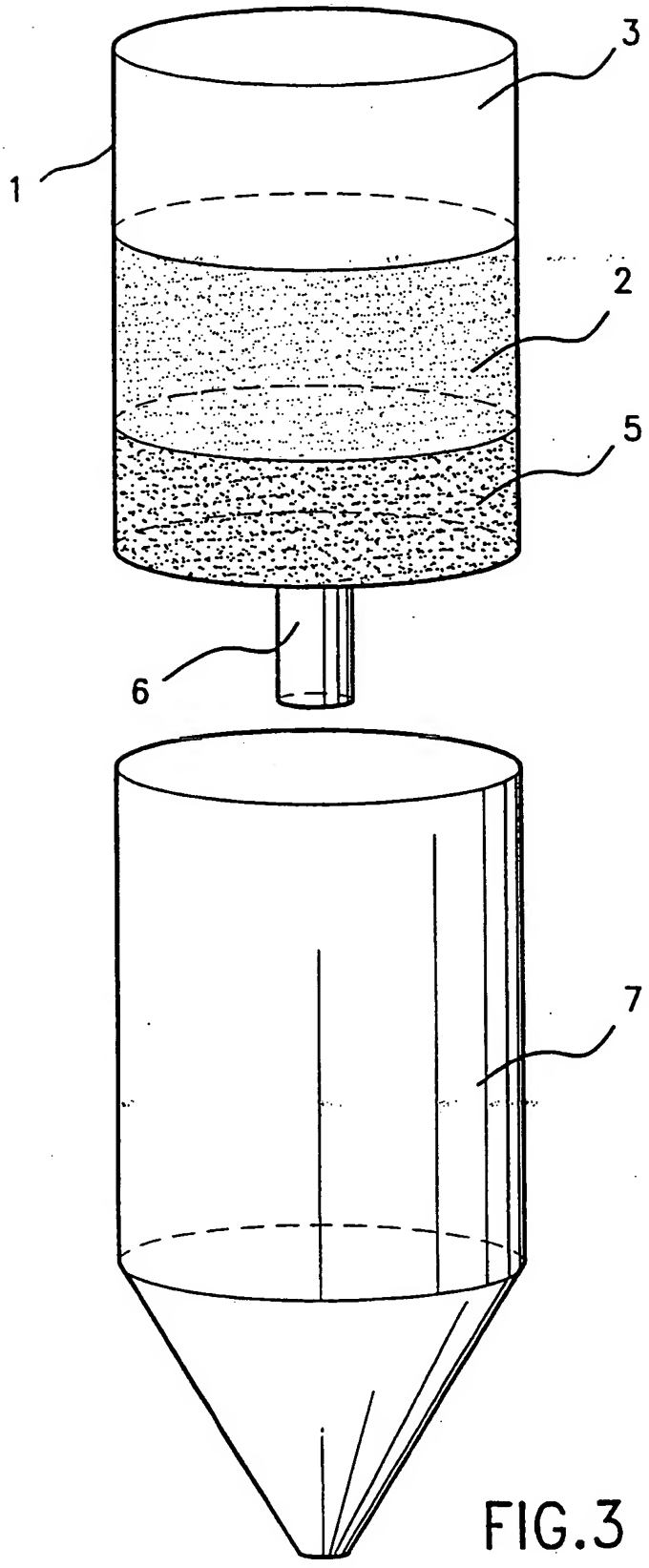


FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/00170

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12N 1/08; C07H 15/12
US CL :435/270; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/270; 536/27, 28

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, BIOSIS, CAPLUS, EMBASE, GENBANK
search terms: purification, isolation, nucleic, matrix, detergent, enzyme, cell, column

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	U S 5,057,426 A (HENCO et al) 15 October 1991, see entire document.	1-19 ------- 20-21
Y	STRATAGENE CATALOG,gene characterization kits, 1988, page 39, see entire document.	20, 21
Y	PROMEGA CATALOG, Wizard Minipreps DNA Purification system. 1993/1994, pages 141-145, see entire document.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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